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Method and device for optimizing a nucleotide sequence for the purpose of expression of a protein

The invention relates generally to the production of synthetic DNA sequences and to the use thereof for producing proteins by introducing these DNA sequences into an expression system, for example into a host organism/a host cell or a system for in vitro expression, any of which expresses the appropriate protein. It relates in particular to methods in which a synthetic nucleotide sequence is optimized for the particular expression system, that is to say for example for an organism/for a host cell, with the aid of a computer.

One technique for the preparation and synthesis of proteins is the cloning and expression of the gene sequence corresponding to the protein in heterologous systems, e.g. Escherichia coli or yeast. Naturally occurring genes are, however, frequently suboptimal for this purpose. Since in a DNA sequence expressing a protein in each case one triplet of bases (codon) expresses one amino acid, it is possible for an artificial DNA sequence for expression of the desired protein to be synthesized and to be used for cloning and expression of the protein. One problem with this procedure is that a predefined amino acid sequence does not correspond to a unique nucleotide sequence. This is referred to as the degeneracy of the genetic code. The frequency with which different organisms use codons for expressing an amino acid differs (called the codon usage). There is ordinarily in a given organism one codon which is predominantly used and one or more codons which are used with comparatively low frequency by the organism for expressing the corresponding amino acid. Since the synthesized nucleotide sequence is to be used in a particular organism, the choice of the codons ought to be adapted to the codon usage of the appropriate organism. A further important variable is the GC content (content of the bases guanine and cytosine in a sequence). Further factors which may influence the result of expression are DNA motifs and repeats or inverse complementary repeats in the base sequence. Certain base sequences produce in a given organism certain functions which may not be desired within a coding sequence. Examples are cis-active sequence motifs such as splice sites or transcription terminators. The unintentional presence of a particular motif may reduce or entirely suppress expression or even have a toxic effect on the host organism. Sequence repeats may lead to lower genetic stability and impede the synthesis of repetitive segments owing to the risk of incorrect hybridizations. Inverse complementary repeats may lead to the formation of unwanted secondary structures at the RNA level or cruciform structures at the DNA level, which impede transcription and lead to genetic instability, or may have an adverse effect on translation efficiency.

A synthetic gene ought therefore to be optimized in relation to the codon usage and the GC content and, on the other hand, substantially avoid the problems associated with DNA motifs and sequence repeats and inverse complementary sequence repeats. These requirements cannot, however, ordinarily be satisfied simultaneously and in an optimal manner. For example, optimization to optimal codon usage may lead to a highly repetitive sequence and a considerable difference from the desired GC content. The aim therefore is to reach a compromise which is as optimal as possible between satisfying the various requirements. However, the large number of amino acids in a protein leads to a combinatorial explosion of the number of possible DNA sequences which - in principle - are able to express the desired protein. For this reason, various computer-assisted methods have been proposed for ascertaining an optimal codon sequence.

P.S. Sarkar and Samir K. Brahmachari, Nucleic Acids Research **20** (1992) 5713 describe investigations into the role of the choice of codons in the formation of certain spatial structures of a DNA sequence. This involved generation of all the possible degenerate nucleotide sequences. Assessment of the sequences in relation to the presence of structural motifs and to structure-forming segments was performed by a computer using a knowledge base. The use of a quality function is not disclosed.

D.M. Hoover and J. Lubkowski, Nucleic Acid Research **30** (2002), No.10 e43 proposes a computer-assisted method in which the nucleotide sequence is divided into an odd number of segments for each of which a quality function (score) is calculated. The quality function includes inter alia the codon usage, the possibility of forming hairpin structures and the differences from the desired melting temperature. The value of the quality function for the complete sequence is determined from the total of the values of the quality function for the individual segments. The codon occupation within a segment is optimized by a so-called Monte-Carlo method. This entails random selection of codon positions in which the codon of an initial sequence is replaced by a randomly selected equivalent codon. At the same time, the limits of the segments are redefined in an iteration. In this way there is random generation of a complete gene sequence. If the value of the quality function for the complete sequence is less than the previous sequence, the new sequence is retained. If it is larger, the new sequence is retained with a certain probability, this probability being controlled by a Boltzmann statistic. If the sequence does not change during a predetermined number of iterations, this sequence is regarded as optimal sequence.

Random methods of this type have the disadvantage that they depend greatly on the choice of the convergence criteria..

It is the object of the invention to provide an alternative method for optimizing a nucleotide sequence for the expression of a protein on the basis of the amino acid sequence of the protein, which can be implemented with relatively little storage space and relatively little computing time on a computer, and which avoids in particular the disadvantages of the random methods.

This object is achieved according to the invention by a method for optimizing a nucleotide sequence for the expression of a protein on the basis of the amino acid sequence of the protein, which comprises the following steps carried out on a computer:

- generation of a first test sequence of n codons which correspond to n consecutive amino acids in the protein sequence, where n is a natural number and is less than or equal to N, the number of amino acids in the protein sequence,
- specification of m optimiziation positions in the test sequence which correspond to the position of m codons, in particular of m consecutive codons, at which the occupation by a codon, relative to the test sequence, is to be optimized, where $m \leq n$ and $m < N$,
- generation of one or more further test sequences from the first test sequence by replacing at one or more of the m optimization positions a codon of the first test sequence by another codon which expresses the same amino acid,
- assessment of each of the test sequences with a quality function and ascertaining the test sequence which is optimal in relation to the quality function,

- specification of p codons of the optimal test sequence which are located at one of the m optimization positions, as result codons which form the codons of the optimized nucleotide sequence at the positions which corresponds to the position of said p codons in the test sequence, where p is a natural number and p ≤ m,
- iteration of the preceding steps, where in each iteration step the test sequence comprises the appropriate result codon at the positions which correspond to positions of specified result codons in the optimized nucleotide sequence, and the optimization positions are different from positions of result codons.

According to the preferred embodiment of the invention, the aforementioned steps are iterated until all the codons of the optimized nucleotide sequence have been specified, i.e. occupied by result codons.

Thus, the optimization according to the invention is not of the sequence as a whole but successively on part regions. The p result codons specified as optimal in one iteration step are not changed again in the subsequent iteration steps and, on the contrary, are assumed to be given in the respective optimization steps. It is preferred for the number of result codons which are specified in this way for further iterations and are treated as predefined to be smaller than the number m of optimization positions at which the codons are varied in an iteration step. In at least the majority of iteration steps and, in a particular embodiment, in all iteration steps apart from the first, in turn m is smaller than the number of codons of the test sequence (n). This makes it possible to take account not only of local effects on the m varied positions, but also of wider-ranging correlations, e.g. in connection with the development of RNA secondary structures.

According to the embodiments preferred at present, m is in the range from 3 to 20, preferably in the range from 5 to 10.. With this choice of this parameter it is possible to vary the codons with an acceptable usage of storage and computing time and, at the same time, achieve good optimization of the sequence.

According to one embodiment, m need not be the same in the various iteration steps but, on the contrary, may also be different in different iteration steps. It is also possible to provide for variation of the test sequence for different values of m to be carried out in one iteration step and, where appropriate, for taking account only of the optimization result for one value of m, in order to reduce influences of the quantity m on the optimization result, and in order to check whether an increase in the number m leads to a change in the result.

According to the preferred embodiment, the m optimization positions or at least some of them are connected and thus form a variation window, on which the codon occupation is varied, in the test sequence.

The invention can in particular provide for some of the m optimization positions on which the codons are varied to be identical in two or more consecutive iteration steps. If the m positions are connected, this means that the variation window in one iteration step overlaps with the variation window of a preceding iteration step.

The invention can provide for the m optimization positions of the test sequences in one or more iteration steps to follow directly one or more result codons which have been specified as part of the optimized nucleotide sequence.

The invention can likewise provide for the p codons which are specified as result codons of the optimized nucleotide sequence in one or more iteration steps to be p consecutive codons which preferably directly follow one or more result codons which have been specified as part of the optimized nucleotide sequence in an earlier step.

The invention can provide for the nucleotide sequence to be optimized from one of its ends. In particular, the invention can provide for an increase in each iteration step of the length of the test sequence of the previous iteration step by a particular number of codons, which may be different in different iterations, until n = N. If n = N and the number of positions in the test sequence not occupied by result codons is smaller than or equal to the value of m used in the preceding iterations, or if this number on use of different values of m in different iterations is in the region of the values of m in question, it is possible to set p = m in the corresponding iteration step, where m is at the same time the number of codons not yet specified. The occupation which is found to be optimal for the optimization positions is then accepted for the result codons at these optimization positions. This applies in particular when a test sequence is generated for every possible combination of occupations of the optimization positions.

However, it is also possible to provide for the region of the test sequence within the complete sequence in one iteration step not, or not completely, to include the region of a test sequence in a previous iteration step. For example, the test sequence itself may form a window on the complete sequence, e.g. a window of fixed length, which window is shifted on the complete sequence during the various iterations.

According to a preferred embodiment, the test sequence is extended after each step by p codons, it being possible in particular for m to be constant for all iteration steps.

In analogy to the embodiment of the invention described above, it is also possible to provide for the nucleotide sequence to be optimized from a site in its interior. This can take place for example in such a way that an initial test sequence corresponding to a region in the interior of the nucleotide sequence to be optimized is initially enlarged successively on one side until the end of the nucleotide sequence to be optimized or another predefined point is reached on the nucleotide sequence to be optimized, and then the test sequence is enlarged towards the other side until the other end of the nucleotide sequence to be optimized or another predetermined point is reached there on the nucleotide sequence to be optimized.

The invention can also provide for the test sequences in one iteration step to consist of an optimized or otherwise specified partial sequence of length q and two variation regions which are connected on both sides thereof and have a length of respectively m_1 and m_2 codons, where $q + m_1 + m_2 = n$. The occupation of the variation regions can be optimized for both variation regions together by simultaneously varying and optimizing the codons on the m_1 and m_2 locations. It is preferred in such a case for p_1 and p_2 codons in the first and second variation region, which are used as given basis for the further iteration, to be specified in each iteration step. However, it is also possible to provide for the two variation regions to be varied and optimized independently of one another. For example, it is possible to provide for the occupation to be varied in only one of the two variation regions, and for codons to be specified only in the one region, before the variation and optimization in the second region takes place. In this case, the p_1 specified codons in the first region are assumed as given in the optimization of the second region. This procedure is worthwhile when small correlations at the most are to be expected between the two regions.

According to this embodiment, it is possible to provide for the nucleotide sequence to be optimized starting from a point or a region in the interior of the sequence.

The invention can provide in particular for the region of the test sequence on the complete sequence in each iteration step to include the region of the test sequences in all the preceding iteration steps, and for the region of a test sequence in at least some of the preceding iteration steps to be located in each case in the interior or in each case at the border of the region of the test sequence in the current iteration step.

The invention can provide for the nucleotide sequence to be optimized independently on different part regions. The optimized nucleotide sequence can then be the combination of the different optimized partial sequences. It is also possible to provide for at least some of the respective result codons from two or more optimized part regions to be used as constituent of a test sequence in one or more iterations.

A preferred embodiment of the invention provides for test sequences with all possible codon occupations for the m optimization positions to be generated in one iteration step from the first test sequence, and the optimal test sequence to be ascertained from all possible test sequences in which a codon at one or more of the m optimization positions has been replaced by another codon which expresses the same amino acid.

According to one embodiment of the invention, the quality function used to assess the test sequences is the same in all or at least the majority of the iterations. The invention may, however, also provide for different quality functions to be used in different iterations, for example depending on the length of the test sequences.

The method of the invention may comprise in particular the following steps:

- assessment of each test sequence with a quality function,
- ascertaining of an extreme value within the values of the quality function for all partial sequences generated in an iteration step,
- specification of p codons of the test sequence which corresponds to the extremal value of the weight function as result codons at the appropriate positions, where p is a natural number and p ≤ m.

The quality function can be defined in such a way that either a larger value of the quality function means that the sequence is nearer the optimum, or a smaller value means that it is nearer the optimum. Correspondingly, the maximum or the minimum of the quality function among the generated codon sequences will be ascertained in the step of ascertaining the extreme value.

The invention can provide for the quality function to take account of one or more of the following criteria: codon usage for a predefined organism, GC content, sequence motifs, repetitive sequences, secondary structures, inverse repeats.

The invention can provide in particular for the quality function to take account of one or more of the following criteria:

- cis-active sequence motifs, especially DNA/protein interaction binding sites and RNA/protein interaction binding sites, preferably splice motifs, transcription factor binding sites, transcription terminator binding sites, polyadenylation signals, endonuclease recognition sequences, immunomodulatory DNA motifs, ribosome binding sites, recognition sequences for recombination enzymes, recognition signals for DNA-modifying enzymes, recognition sequences for RNA-modifying enzymes, sequence motifs which are underrepresented in a predefined organism.

The invention can also provide for the quality function to take account of one or more of the following criteria:

- exclusion or substantial exclusion of inverse complementary sequence identities of more than 20 nucleotides to the transcriptome of a predefined organism,
- exclusion or substantial exclusion of homology regions of more than 1000 base pairs, preferably 500 base pairs, more preferably 100 base pairs, to a predefined DNA sequence, for example to the genome of predefined organism or to the DNA sequence of a predefined vector construct.

The first of the two criteria relates to the exclusion of the mechanism known as RNA indifference, with which an organism eliminates or deactivates RNA sequences with more than 20 nucleotides exactly identical to another RNA sequence. The intention of the second criterion is to prevent the occurrence of recombination, that is to say incorporation of the sequence into the genetic material of the organism, or mobilization of DNA sequences through recombination with other vectors. Both criteria can be used as absolute exclusion criteria, i.e. sequences for which one or both of these criteria are satisfied are not taken into account. The invention can also provide, as explained in more detail below in connection with sequence motifs, for these criteria to be assigned a weight which in terms of contribution is larger than the largest contribution of criteria which are not exclusion criteria to the quality function.

The invention can also, where appropriate together with other criteria, provide the criterion that no homology regions showing more than 90% similarity and/or 99% identity to a predefined DNA sequence, for example to the appropriate genome sequence of the predefined organism or to the DNA sequence of a predefined vector construct, are generated. This criterion can also be implemented either as absolute exclusion criterion or in such a way that it makes a very large contribution, outweighing the contribution of other criteria which are not exclusion criteria, to the quality function.

It is possible to provide in particular for the quality function to be a function of various single terms, in particular a total of single terms, which in each case assess one criterion from the following list of criteria:
codon usage for a predefined organism, GC content, DNA motifs, repetitive sequences, secondary structures, inverse repeats.

Said function of single terms may be in particular a linear combination of single terms or a rational function of single terms. The criteria mentioned need not necessarily be taken completely into account in the weight function. It is also possible to use only some of the criteria in the weight function.

The various single terms in said function are called criterion weights hereinafter.

The invention can provide for the criterion weight relating to the codon usage (CU score) to be proportional to $\sum_i f_{ci}/f_{cmaxi}$, where

- f_{ci} is the frequency of the codon placed at site i of the test sequence for the relevant organism to express the amino acid at site i in the amino acid sequence of the protein to be expressed, and
- f_{cmaxi} is the frequency of the codon which expresses most frequently the amino acid at site i in the corresponding organism.

The measure f_{ci}/f_{cmaxi} is known as the relative adaptiveness (cf. P.M. Sharp, W.H. Li, Nucleic Acids Research **15** (3) (1987), 1281 to 1295).

The local weight of the most frequently occurring codon is in this case, irrespective of the absolute frequency with which this codon occurs, set at a particular value, for example 1. This avoids the positions at which only a few codons are available for selection making a greater contribution to the total weight than those at which a larger number of codons are available for selection for expression of the amino acid. The index i may run over the entire n codons of the test sequence or a part thereof. In particular, it is possible to provide in one embodiment for i to run only over the m codons of the optimization positions.

The invention can provide for the criterion weight relating to the codon usage to be used only for the m ordering positions.

It is possible to use instead of the relative adaptiveness also the so-called RSCU (relative synonymous codon usage; cf. P.M. Sharp, W.H. Li, loc. cit.). The RSCU for a codon position is defined by

$$RSCU_{ci} = f_{ci}d_i/(\sum_c f_{ci})$$

where the sum in the denominator runs over all the codons which express the amino acid at site i, and where d_i indicates the number of codons which express said amino acid. In order to define a criterion weight on the basis of the RSCU it is possible to provide for the RSCU to be summed for the respective test sequence over all the codons of the test sequence or a part thereof, in particular over the m codons of the optimization positions. The difference from the criterion weight derived from the relative adaptiveness is that with this weighting each codon position is weighted with the degree of degeneracy, d_i, so that positions at which more codons are available for selection participate more in the criterion weight than positions at which only a few codons or even only a single codon are available for selection.

With the criterion weights described above for the codon usage, the arithmetic mean was formed over the local weights (relative adaptiveness, RSCU).

It can also be provided for the criterion weight relating to the codon usage to be proportional to the geometric mean of the local relative adaptiveness or the local RSCU, so that the following therefore applies

$$\text{CUScore} = K(\Pi_i \ \text{RSCU}_i)^{1/L}$$

or

$$\text{CUScore} = K(\Pi_i f_{ci}/f_{cmaxi})^{1/L}$$

where K is a scaling factor, and L is the number of positions over which the product is formed. Once again, it is possible in this case to form the product over the complete test sequence or a part, in particular over the m optimization positions.

In this connection, the invention also provides a method for optimizing a nucleotide sequence for expression of a protein on the basis of the amino acid sequence of the protein, which comprises the following steps carried out on a computer:

- generation of one or more test sequences of n codons which correspond to n consecutive amino acids in the protein sequence, where n is a natural number less than or equal to N, the number of amino acids in the protein sequence,
- assessment of the one or more test sequences on the basis of a quality function which comprises a geometric or arithmetic mean of the relative adaptiveness or of the RSCU over a number of L codon positions, where L is less than or equal to N,
- generation of one or more new test sequences depending on the result of said assessment.

It is moreover possible for the generation of one or more new test functions in the manner described above to take place in such a way that the new test sequences comprise a particular number of result codons specified on the basis of the preceding iterations but, for example, also in such a way that a particular test sequence is used with a particular probability, which depends on the value of the quality function, as basis for further iterations, in particular the further generation of test sequences, as is the case with Monte-Carlo methods.

Whereas the quality of a codon in the abovementioned methods is defined through the frequency of use in the transcriptome or a gene reference set of the expression organism, the quality of a particular codon can also alternatively be described by the biophysical properties of the codon itself. Thus, for example, it is known that codons with an average codon-anticodon binding energy are translated particularly efficiently.

It is therefore possible to use as measure of the translational efficiency of a test sequence for example the P2 index which indicates the ratio of the frequency of codons with average binding energy and codons with extremely strong or weak binding energy. It is also possible alternatively to utilize data obtained experimentally or by theoretical calculations for the translational efficiency or translation accuracy of a codon for the quality assessment. The abovementioned assessment criteria may be advantageous especially when the tRNA frequencies of the expression system need not be taken into account, because they can be specified by the experimentor as, for example, in in vitro translation systems.

The invention can provide for the criterion weight relating to the GC content (GCScore) to be a function of the contribution of the difference of the ascertained GC content of the partial sequence, GCC, to the optimal GC content, GCC_{opt}, where the GG content means the relative proportion of guanine and cytosine, for example in the form of a particular percentage proportion.

The criterion weight GCScore can have the following form, in particular:

$$GCScore = |\overline{GCC} - GCC_{opt}|^{g} \cdot h$$

where

$\overline{GCC}$ is the actual GC content of the test sequence or of a predetermined part of the test sequence, GCC, or the average GC content of the test sequence or of a predetermined part of the test sequence, <GCC>,

GCC_{opt} is the desired (optimal) GC content,

g is a positive real number, preferably in the range from 1 to 3, in particular 1.3, h is a positive real number.

The factor h is essentially a weighting factor which defines the relative weight of the criterion weight GCScore vis-à-vis the other criterion weights. Preferably, h is chosen so that the amount of the maximally achievable value of GCScore is in a range from one hundredth of up to one hundred times another criterion weight, in particular all criterion weights which represent no exclusion condition, such as, for example, the weights for a wanted or unwanted sequence motif.

To determine the average GC content it is possible to provide for a local GC content relating to a particular base position to be defined by the GC content on a window which was a particular size and which comprises this base and which, in particular, can be centered on this base. This local GC content is then averaged over the test sequence or a part region of the test sequence, in particular over the m optimization positions, it being possible to use both an arithmetic mean and a geometric mean here too. On use of an average GC content defined in this way there are fewer variations between test sequences differing in length n.

The invention can provide for the GC content to be ascertained over a window which is larger than the region of the m optimization positions and includes this. If the optimization positions form a coherent variation window it is possible to provide for b bases before and/or after the variation window to be included in the determination of the criterion weight for the GC content (GCScore), where b can be in a range from 15 to 45 bases (corresponding to 5 to 15 codons), preferably in a range from 20 to 30 bases.

The invention can further provide, inasmuch as the quality function is maximized, for a fixed amount to be subtracted for each occurrence of a sequence motif which is not permitted or is unwanted, and for a fixed amount to be added for each wanted or required motif, when ascertaining the value of the quality function (and vice versa for minimization of the quality function). This amount for unwanted or required motifs can be distinctly larger than all other criterion weights, so that the other criteria are unimportant compared therewith. An exclusion criterion is achieved thereby, while at the same time there is differentiation according to whether a motif has occurred once or more than once. However, it is likewise possible to define a worthwhile quality function and carry out an assessment of the test sequences with the quality function even if the condition relating to the sequence motif (non-presence of a particular motif/presence of a particular motif) cannot be satisfied for all test sequences produced in an iteration step. This will be the case in particular when the length n of the test sequences is relatively small compared with N, because a particular motif can often occur only when n is relatively large, because of the predefined amino acids of the protein sequence.

The invention can further provide for the complete test sequence or part thereof to be checked for whether particular partial sequence segments or sequence segments similar to particular partial sequence segments occur in another region of the test sequence or of a given region of the test sequence or whether particular partial sequence segments or sequence segments similar to particular partial sequence segments occur in the inverse complementary test sequence or part of the inverse complementary test sequence, and for a criterion weight for sequence repeats (repeats) and/or inverse sequence repeats (inverse repeats) to be calculated dependent thereon.

Ordinarily, the sequence will be checked not only for whether a particular sequence segment is present identically in the test sequence or the inverse complementary test sequence or of a part region thereof, but also for whether a similar, i.e. only partially matching, sequence is present in the test sequence or the inverse complementary test sequence or of a part thereof. Algorithms for finding global matches (global alignment algorithms) or local matches (local alignment algorithms) of two sequences are generally known in bioinformatics. Suitable methods include, for example, the dynamic programming algorithms generally known in bioinformatics, e.g. the so-called Needleman-Wunsch algorithm for global aligment and the Smith-Waterman algorithm for local alignment. In this regard, reference is made for example to Michael S. Waterman, Introduction to Computational Biology, London, New York 2000, especially pages 207 to 209 or Dan Gusfield, Algorithms on Strings, Trees and Sequences, Cambridge, 1999, especially pages 215 to 235.

The invention can in particular provide for every repeat of a partial sequence segment in another part of the test sequence or of a predefined region of the test sequence to be weighted with a particular weight which represents a measure of the degree of match and/or the size of the mutually similar segments, and for the weights of the individual repeats to be added to ascertain the criterion weight relating to the repeats or inverse complementary repeats. It is likewise possible to provide for the weights of the individual repeats to be exponentiated with a predefined exponent whose value is preferably between 1 and 2, and then for the summation to ascertain the criterion weight relating to the repeats or inverse complementary repeats to be carried out. It is moreover possible to provide for repeats below a certain length and/or repeats whose weight fraction is below a certain threshold not to be taken into account. The invention can provide, for the calculation of the appropriate criterion weight, for account to be taken only of the repeats or inverse complementary repeats of a partial sequence segment which is located in a predefined part region of the test sequence (test region), e.g. at its end and/or in a variation window. It is possible to provide for example for only the last 36 bases of the test sequence to be checked for whether a particular sequence segment within these 36 bases matches with another sequence segment of the complete test sequence or of the complete inverse complementary test sequence.

The invention can provide for only the segment or the M segments of the test sequence which provide the largest, or largest in terms of amount, contribution to the criterion weight, where M is a natural number, preferably between 1 and 10, to be taken into account in the criterion weights relating to repeats, inverse complementary repeats and/or DNA motifs.

According to one embodiment of the invention, it is possible to provide for generation of a matrix whose number of columns corresponds to the number of positions of the region of the test sequence (test region) which is to be checked for repeats in other regions, and whose number of rows corresponds to the number of positions of the region of the test sequence with which comparison is intended (comparison region). Both the test region and the comparison region may include the complete test sequence.

The invention can further provide for the total weight function TotScore to be determined as follows:

TotScore = CUScore - GCScore - REPScore - SiteScore

where CUScore is the criterion weight for the codon usage, GCScore is the criterion weight for the GC content, REPScore is the criterion weight for repeats and inverse complementary repeats of identical or similar sequence segments, and SiteScore is the criterion weight for the occurrence of unwanted or required motifs.

The weight REPScore can, according to one embodiment of the invention, consist of a sum of two components, of which the first indicates the criterion weight for the repeat of identical or similar sequence segments in the test sequence itself or of a part region thereof, and the second component indicates the criterion weight for inverse complementary repeats of identical or similar sequence segments in the test sequence or of a part region thereof.

If the quality function is composed of portions of a plurality of test criteria, especially when the quality function consists of a linear combination of criterion weights, a test sequence need not necessarily be assessed according to all criteria in an iteration step. On the contrary, the assessment can be stopped as soon as it is evident that the value of the quality function is less or, speaking more generally, less optimal than the value of the quality function of a test sequence which has already been assessed. In the embodiments described previously, most of the criteria, such as the criterion weights for repetitive elements, motifs to be excluded etc., are included negatively in the quality function. If, after calculating the criterion weights which are included positively in the quality function and, where appropriate, some of the criterion weights which are included negatively in the quality function, the summation corresponding to the linear combination, defined by the quality function, of the appropriate previously calculated criterion weights gives a value which is smaller than a previously calculated value of the complete quality function for another test sequence, the currently assessed test sequence can be eliminated at once. It is likewise frequently possible, for example when a criterion weight is considerably larger in terms of amount than all the other weights, for the assessment to be stopped at once after ascertaining the corresponding criterion weight. If, for example, an unwanted motif has not appeared in a first test sequence, and the unwanted motif appears in a second test sequence, the second test sequence can be immediately excluded, because the criterion weight for the motif search is so large that it cannot be compensated by other criterion weights.

The invention can provide in particular in embodiments in which the quality function can be calculated iteratively for there to be, in at least one iteration, determination of an upper (or in the case of optimization to the minimum of the quality function lower) limit below (or above) which the value of the complete quality function lies, and for the iteration of the quality function to be stopped when this value is below (or above) the value which has previously been ascertained for the complete quality function for a test sequence.

The invention can provide in these cases for said upper or lower limit to be used if necessary as value of the quality function in the further method for this test sequence, and/or for the corresponding test sequence to be eliminated in the algorithm, for example through the variable for the optimized test sequence remaining occupied by a previously found test sequence for which the quality function a higher value than the abovementioned limit, and the algorithm to go on to the assessment of the next test sequence. The invention can moreover, especially when the quality function is a linear combination of criterion weights, provide for calculation in the first iterations of that contribution or those contributions whose highest value or whose minimal value has the highest absolute value.

The invention can provide in the case of a quality function which is optimized to its maximum and which is formed by a linear combination of criterion weights for firstly the positive portions of the linear combination to be calculated and the iteration to be stopped when, in one iteration after the calculation of all positive criterion weights, the value of the quality function in this iteration is smaller than the value of the complete quality function for another test sequence.

The invention can also provide for an iteration of the quality function to be stopped when it is found in an iteration that the sum of the value of the quality function calculated in this iteration and the maximum value of the contribution of the as yet uncalculated criterion weights is below the value of the complete quality function of another test sequence.

The method of the invention may include the step of synthesizing the optimized nucleotide sequence.

It is possible to provide in this connection for the step of synthesizing the optimized nucleotide sequence to take place in a device for automatic synthesis of nucleotide sequences, for example in an oligonucleotide synthesizer, which is controlled by the computer which optimizes the nucleotide sequence.

The invention can provide in particular for the computer, as soon as the optimization process is complete, to transfer the ascertained data concerning the optimal nucleotide sequence to an oligonucleotide synthesizer and cause the latter to carry out the synthesis of the optimized nucleotide sequence.

This nucleotide sequence can then be prepared as desired. The protein is expressed by introducing the appropriate nucleotide sequence into host cells of a host organism for which it is optimized and which then eventually produces the protein.

The invention also provides a device for optimizing a nucleotide sequence for the expression of a protein on the basis of the amino acid sequence of the protein, which has a computer unit which comprises:

- a unit for generation of a first test sequence of n codons which correspond to n consecutive amino acids in the protein sequence, where n is a natural number less than or equal to N, the number of amino acids in the protein sequence,
- a unit for specification of m optimiziation positions in the test sequence which correspond to the position of m codons at which the occupation by a codon, relative to the test sequence, is to be optimized, where $m \leq n$ and $m < M$,
- a unit for generation of one or more further test sequences from the first test sequence by replacing at one or more of the m optimization positions a codon of the first test sequence by another codon which expresses the same amino acid,
- a unit for assessment of each of the test sequences with a quality function and for ascertaining the test sequence which is optimal in relation to the quality function,
- a unit for specification of p codons of the optimal test sequence which are located at one of the m optimization positions, as result codons which form the codons of the optimized nucleotide sequence at the positions which correspond to the positions of said p codons in the test sequence, where p is a natural number and $p \leq m$,
- a unit for iteration of the steps of generation of a plurality of test functions, of assessment of the test sequences and of specification of result codons, preferably until all the codons of the optimized nucleotide sequence have been specified, where in each iteration step the test sequence

comprises the appropriate result codon at the positions which correspond to positions of specified result codons in the optimized nucleotide sequence, and the optimization positions are different from positions of result codons.

The aforementioned units need not be different but may, in particular, be implemented by a single device which implements the functions of the aforementioned units.

The device of the invention may generally have a unit for carrying out the steps of the methods described above.

The device of the invention may have an oligonucleotide synthesizer which is controlled by the computer so that it synthesizes the optimized nucleotide sequence.

In this embodiment of the invention, the optimized nucleotide sequence can be synthesized either automatically or through an appropriate command from the user, without data transfers, adjustment of parameters and the like being necessary.

The invention also provides a computer program which comprises program code which can be executed by a computer and which, when it is executed on a computer, causes the computer to carry out a method of the invention.

The program code can moreover, when it is executed on a computer, cause a device for the automatic synthesis of nucleotide sequences to prepare the optimized nucleotide sequence.

The invention also provides a computer-readable data medium on which a program of the invention is stored in computer-readable form.

The invention further provides a nucleic acid which has been or can be prepared by a method of the invention, and a vector which comprises such a nucleic acid. The invention further provides a cell which comprises such a vector or such a nucleic acid, and a non-human organism or a non-human life form which comprises such a cell, it also being possible for such a non-human life form to be mammal.

Whereas in random methods there is no correlation between a sequence in a preceding iteration step and the sequence in a subsequent iteration step, there is according to the invention new specification of a codon in each iteration step. Since the test sequence is varied on only part of the complete sequence, the method can be carried out with less effort. It is possible in particular to evaluate all possible combinations of codons in the variation region. The invention makes use in an advantageous manner of the circumstance that long-range correlations within a nucleotide sequence are of minor importance, i.e. that to achieve an acceptable optimization result it is possible to vary the codons at one position substantially independently of the codons at a more remote position.

The method of the invention makes it possible to a greater extent than previous methods for relevant biological criteria to be included in the assessment of a test sequence. For example, with the method of the invention it is possible to take account of wanted or unwanted motifs in the synthetic nucleotide sequence. Since in a motif search even an individual codon may be crucial for whether a particular motif is present or not, purely stochastic methods will provide optimized sequences which comprise a required motif only with a very low probability or not at all. However, this is possible with the method of the invention because all codon combinations are tested over a part region of the sequence. It is possible where appropriate in order to ensure the presence or non-presence of a particular sequence motif to make the number m of optimization positions so large that it is larger than the number of codon positions (or the number of base positions divided by 3) of the corresponding motif. If the m optimization positions are connected, it is thus ensured that the occurrence of a particular sequence motif can be reliably detected and the corresponding motif can be ensured in the sequence or excluded from the latter. The numerical calculation of the quality function has particular advantages on use of weight matrix scans. Since in this case a different level of importance for recognition or biological activity can be assigned to the different bases of a recognition sequence, it is possible in the method of the invention, in which all possible codon combinations are tested over a part region of the sequence, to find the sequence which, for example, switches off most effectively a DNA motif by eliminating the bases which are most important for the activity, or it is possible to find an optimized compromise solution with inclusion of other criteria.

The invention is not in principle restricted to a particular organism. Organisms for which an optimization of a nucleotide sequence for expression of a protein using the method of the invention is of particular interest are, for example, organisms from the following groups:

- viruses, especially vaccinia viruses,
- prokaryotes, especially Escherichia coli, Caulobacter cresentus, Bacillus subtilis, Mycobacterium spec.,
- yeasts, especially Saccharomyces cerevisiae, Schizosaccharomyces pombe, Pichia pastoris, Pichia angusta,

- insects, especially Sprodoptera frugiperda, Drosophila spec.,
- mammals, especially Homo sapiens, Macaca mulata, Mus musculus, Bos taurus, Capra hircus, Ovis aries, Oryctolagus cuniculus, Rattus norvegicus, Chinese hamster ovary,
- monocotyledonous plants, especially Oryza sativa, Zea mays, Triticum aestivum,
- dicotyledonous plants, especially Glycin max, Gossypium hirsutum, Nicotiana tabacum, Arabidopsis thaliana, Solanum tuberosum.

Proteins for which an optimized nucleotide sequence can be generated using the method of the invention are, for example:

- enzymes, especially polymerases, endonucleases, ligases, lipases, proteases, kinases, phosphatases, topoisomerases,
- cytokines, chemokines, transcription factors, oncogenes,
- proteins from thermophilic organisms, from cryophilic organisms, from halophilic organisms, from acidophilic organisms, from basophilic organisms,
- proteins with repetitive sequence elements, especially structural proteins,
- human antigens, especially tumor antigens, tumor markers, autoimmune antigens, diagnostic markers,
- viral antigens, especially from HAV, HBV, HCV, HIV, SIV, FIV, HPV, rinoviruses, influenza viruses, herpesviruses, poliomaviruses, hendra virus, dengue virus, AAV, adenoviruses, HTLV, RSV,
- antigens of protozoa and/or disease-causing parasites, especially those causing malaria, leishmania; trypanosoma, toxoplasmas, amoeba,
- antigens of disease-causing bacteria or bacterial pathogens, especially of the genera Chlamydia, staphylococci, Klebsiella, Streptococcus, Salmonella, Listeria, Borrelia, Escherichia coli,

- antigens of organisms of safety level L4, especially Bacillus anthracis, Ebola virus, Marburg virus, poxviruses.

The preceding list of organisms and proteins for which the invention is used is by no means restrictive and is intended merely as example for better illustration.

Further features and advantages of the invention are evident from the following description of exemplary embodiments of the invention with reference to the appended drawings.

Figures 1a, 1b show a flow diagram of an exemplary embodiment of the method of the invention,

Figure 2 illustrates the ratio of test sequence, optimized DNA sequence, combination DNA sequence and amino acid sequence for an exemplary embodiment of the invention,

Figure 3 shows the regions for determining the sequence repeat,

Figure 4a and 4b show diagrammatically a scheme for determining sequence repeats,

Figure 5a shows the codon usage on exclusive optimization for codon usage,

Figure 5b shows the GC content on exclusive optimization for codon usage,

Figure 6a shows the codon usage on use of a first quality function,

Figure 6b shows the GC content on use of a first quality function, Figure 7a shows the codon usage on use of a second quality function,

Figure 7b shows the GC content on use of a second quality function,

Figure 8a shows the codon usage on use of a third quality function,

Figure 8b shows the GC content on use of a third quality function,

Fig. 9 shows a representative murine MIP1alpha calibration line in connection with example 3,

Fig. 10 illustrates the percentage increase in the total amount of protein after transfection of synthetic expression constructs compared with wild-type expression constructs in connection with example 3,

Fig. 11 shows a representative ELISA analysis of the cell lysates and supernatants of transfected H1299 cells in connection with example 3 and

Fig. 12A to 12C shows the expression analysis of the synthetic reading frames and of the wild-type reading frames in connection with example 3.

According to a preferred embodiment of the invention, in one iteration the choice of the codon for the ith amino acid of an amino acid sequence of length N is considered. For this purpose, all possible codon combinations of the available codons for the amino acids at positions i to i + m - 1 are formed. These positions form a variation window and specify the optimization positions at which the sequence is to be varied. Every combination of codons on this variation window results in a DNA sequence with 3 m bases, which is called combination DNA sequence (CDS) hereinafter. In each iteration step, a test sequence which comprises the CDS at its end is formed for each CDS. In the first iteration step, the test sequences consist only of the combination DNA sequences. The test sequences are weighted with a quality function which is described in detail below, and the first codon of the CDS which exhibits the maximum value of the quality function is retained for all further iterations as codon of the optimized nucleotide sequence (result codon). This means that when the ith codon has been specified in an iteration, each of the test sequences comprises in the next iteration this codon at position i, and the codons of the various combination DNA sequences at positions i + 1 to i + m. Thus, in the jth iteration, all test sequences consist at positions 1 to j - 1 of the codons found to be optimal in the preceding iterations, while the codons at positions j to j + m - 1 are varied. The quality of the DNA sequence can be expressed as criterion weight (individual score) for each individual test criterion. A total weight (total score) is formed by adding the criterion weights weighted according to specifications defined by the user and indicates the value of the quality function for the complete test sequence. If j = N - m + 1, the optimal test sequence is at the same time the optimized nucleotide sequence according to the method of the invention. All the codons of the optimal CDS in this (last) step are therefore specified as codons of the optimized nucleotide sequence.

The procedure described above is illustrated diagrammatically in figure 1. The algorithm starts at the first amino acid (i=1). A first CDS of the codons for amino acids i to i + m - 1 is then formed (in the first iteration, these are amino acids 1 to m). This CDS is combined with the previously optimized DNA sequence to give a test sequence. In the first step, the optimized DNA sequence consists of 0 elements. The test sequence therefore consists in the first iteration only of the previously formed (first) CDS.

The test sequence is then evaluated according to criteria defined by the user. The value of a quality function is calculated by criterion weights being calculated for various assessment criteria and being calculated in an assessment function. If the value of the quality function is better than a stored value of the quality function, the new value of the quality function is stored. At the same time, the first codon of the relevant CDS which represents amino acid i is also stored. If the value of the quality function is worse than the stored value, no action is taken. The next step is to check whether all possible CDS have been formed. If this is not the case, the next possible CDS is formed and combined with the previously optimized DNA sequence to give a new test sequence. The steps of evaluating, determining a quality function and comparing the value of the quality function with a stored value are then repeated. If, on the other hand, all possible CDS have been formed, and if $i \neq N - m + 1$, the stored codon is attached at position i to the previously formed optimized DNA sequence. In the first iteration, the optimized DNA sequence is formed by putting the stored codon on position 1 of the optimized DNA sequence. The process is then repeated for the next amino acid (i + 1). If, on the other hand, $i = N - m + 1$, the complete CDS of the optimal test sequence is attached to the optimized DNA sequence previously formed, because it is already optimized in relation to the assessment criteria. Output of the optimized sequence then follows.

The relationship of the various regions is depicted diagrammatically in figure 2. The combination DNA sequence and the region of the previously specified optimized DNA sequence are evident.

The parameter m can be varied within wide limits, the aim being to maximize the number of varied codons for the purpose of the best possible optimization. A worthwhile optimization result can be achieved within an acceptable time with a size of the variation window of from m = 5 to m = 10 using the computers currently available.

Besides the individual weighting of the criterion weights, it is possible to define both the total weight and the criterion weights by suitable mathematical functions which are modified compared with the simple relations such as difference or proportion, e.g. by segmentally defined functions which define a threshold value, or nonlinear functions. The former is worthwhile for example in assessing repeats or inverse complementary repeats which are to be taken into account only above a certain size. The latter is worthwhile for example in assessing the codon usage or the CG content.

Various examples of weighting criteria which can be used according to the invention are explained below without the invention being restricted to these criteria or the weighting functions described below.

Adaptation of the codon usage of the synthetic gene to the codon usage of the host organism is one of the most important criteria in the optimization. It is necessary to take account in this case of the different degeneracy of the various codons (one-fold to six-fold). Quantities suitable for this purpose are, for example, the RSCU (relative synonymous codon usage) or relative frequencies (relative adaptiveness) which are standardized to the frequency of the codon most used by the organism (the codon used most thus has the codon usage of 1), cf. P.M. Sharp, W.H. Li, Nucleic Acid Research **15** (1987), 1281 to 1295.

To assess a test sequence in one embodiment of the invention, the average codon usage is used on the variation window.

When assessing the GC content, a minimal difference in the average GC content from the predefined desired GC content is necessary. An additional aim should be to keep the variations in the GC content over the course of the sequence small.

To evaluate a test sequence, the average percentage GC content of that region of the test sequence which includes the CDS and bases which are located before the start of the CDS and whose number b is preferably between 20 and 30 bases is ascertained. The criterion weight is ascertained from the absolute value of the difference between the desired GC content and the GC content ascertained for the test sequence, it being possible for this absolute value to enter as argument into a nonlinear function, e.g. into an exponential function.

If the variation window has a width of more than 10 codon positions, variations in the GC content within the CDS may be important. In these cases, as explained above, the GC content for each base position is ascertained on a window which is aligned in a particular way in relation to the base position and may include a particular number of, for example 40, bases, and the absolute values of the difference between the desired GC content and the "local" GC content ascertained for each base position are summed. Division of the sum by the number of individual values ascertained results in the average difference from the desired GC content as criterion weight. In the procedure described above it is possible for the location of the window to be defined so that said base position is located for example at the edge or in the center of the window. An alternative possibility is also to use as criterion the absolute amount of the difference between the actual GC content in the test sequence or on a part region thereof to the desired GC content or the absolute amount of the difference between the average of the abovementioned "local" GC content over the test sequence or a part thereof and the desired GC content as criterion. In a further modification it is also possible to provide for the appropriate criterion weight to be used proportionally to the square of the difference between the actual GC content and the desired GC content, the square of the difference between the GC content averaged over the base positions and the desired GC content or the average of the square of the differences between the local GC content and the desired GC content as criterion. The criterion weight for the GC content has the opposite sign to the criterion weight for the codon usage.

Local recognition sequences or biophysical characteristics play a crucial role in cell biology and molecular biology. Unintended generation of corresponding motifs inside the sequence of the synthesized gene may have unwanted effects. For example, the expression may be greatly reduced or entirely suppressed; an effect toxic for the host organism may also arise. It is therefore desirable in the optimization of the nucleotide sequence to preclude unintended generation of such motifs. In the simplest case, the recognition sequence can be represented by a well-characterized consensus sequence (e.g. restriction enzyme recognition sequence) using appropriate IUPAC base symbols. Carrying out a simple regular expression search within the test sequence results in the number of positions found for calculating the appropriate weight. If a certain number of imperfections (mismatches) is permitted, the number of imperfections in a recognized match must be taken into account when ascertaining the weight function, for example by the local weight for a base position being inversely proportional to the number of bases which are assigned to an IUPAC consensus symbol. However, in many cases the consensus sequence is not sufficiently clear (cf., for example, K. Quandt et al., Nucleic Acid Research **23** (1995), 4878). It is possible in such cases to have recourse to a matrix representation of the motifs or use other recognition methods, e.g. by means of neural networks.

In the preferred embodiment of the invention, a value between 0 and 1 which, in the ideal case, reflects the binding affinity of the (potential) site found or its biological activity or else its reliability of recognition is determined for each motif found. The criterion weight for DNA motifs is calculated by multiplying this value by a suitable weighting factor, and the individual values for each match found are added.

The weight for unwanted motifs is included with the opposite sign to that for the codon usage in the overall quality function.

It is possible in the same way to include in the weighting the presence of certain wanted DNA motifs, e.g. RE cleavage sites, certain enhancer sequences or immunostimulatory or immunosuppressive CpG motifs. The weight for wanted DNA motifs is included with the same sign as the weight for the codon usage in the overall assessment.

Highly repetitive sequence segments may, for example, lead to low genetic stability. The synthesis of repetitive segments is also made distinctly difficult because of the risk of faulty hybridization. According to the preferred embodiment of the invention, therefore, the assessment of a test sequence includes whether it comprises identical or mutually similar sequence segments at various points. The presence of corresponding segments can be established for example with the aid of a variant of a dynamic programming algorithm for generating a local alignment of the mutually similar sequence segments. It is important in this embodiment of the invention that the algorithm used generates a value which is suitable for quantitative description of the degree of matching and/or the length of the mutually similar sequence segments (alignment weight). For further details relating to a possible algorithm, reference is made to the abovementioned textbooks by Gusfield or Waterman and M.S. Waterman, M. Eggert, J. Mol. Biology, (1987) 197, 723 to 728.

To calculate the criterion weight relating to the repetitive elements, the individual weights of all the local alignments where the alignment weight exceeds a certain threshold value are summed. Addition of these individual weights gives the criterion weight which characterizes the repetitiveness of the test sequence.

In a modification of the embodiment described above, only the one region which includes the variation window, and a certain number of further bases, e.g. 20 to 30, at the end of the test sequence is checked for whether a partial segment of the test sequence occurs in identical or similar way in this region of another site of the test sequence. This is depicted digrammatically in figure 3. The full line in the middle represents the complete test sequence. The upper line represents the CDS, while the lower region represents the comparison region of the test sequence, which is checked for matching sequence segments with the remainder of the test sequence. The checking of the test sequences for matching or similar segments of the comparison region (cf. figure 3) using the dynamic programming matrix technique is illustrated in figure 4 and 4b. Figure 4a shows the case where similar or matching sequence segments A and B are present in the comparison region itself. Figure 4b shows the case where a sequence segment B in the comparison region matches or is similar to a sequence segment A outside the comparison region.

As alternative to the summation of individual weights it is also possible to provide for only the alignment which leads to the highest individual weight or, more generally only the alignments with the m largest individual weights, to be taken into account.

With the weighting described above it is possible to include both similar sequences which are present for example at the start and at the end of the test sequence, and so-called tandem repeats where the similar regions are both located at the end of the sequence.

Inverse complementary repeats can be treated in the same way as simple repeats. The potential formation of secondary structures and the RNA level or cruciform structures at the DNA level can be recognized on the test sequence by the presence of such inverse complementary repeats (inverse repeats). Cruciform structures at the DNA level may impede translation and lead to genetic instability. It is assumed that the formation of secondary structures at the RNA level has adverse effects on translation efficiency. In this connection, inverse repeats of particular importance are those which form hairpin loops or cruciform structures. Faulty hybridizations or hairpin loops may also have adverse effects in the synthesis of the former from oligonucleotides.

The checking for inverse complementary repeats in principle takes place in analogy to the checking for simple repeats. The test sequence or the comparison region of the test sequence is, however, compared with the inverse complementary sequence. In a refinement, the thermodynamic stability can be taken into account in the comparison (alignment), in the simplest case by using a scoring matrix. This involves for example giving higher weight to a CC or GG match, because the base pairing is more stable, than to a TT or AA match. Variable weighting for imperfections (mismatches) is also possible correspondingly. More specific weighting is possible by using nearest neighbor parameters for calculating the thermodynamic stability, although this makes the algorithm more complex. Concerning a possible algorithm, reference is made for example to L. Kaderali, A. Schliep, Bioinformatics **18** (10) 2002, 1340 to 1349.

For all the assessment criteria, the invention can provide for the corresponding weighting function to be position-dependent. For example, a larger weight can be given to the generation of an RE cleavage sequence at a particular site, or a larger weight can be given to secondary structures at the 5′ end, because they show stronger inhibition there. It is likewise possible to take account of the codon context, i.e. the preceding or following codon(s). It is additionally possible to provide for certain codons whose use at the domain limits plays a role in cotranslational protein folding to make a contribution to the quality function, which contribution depends on whether this codon is nearer to the domain limit or not. Further criteria which may be included in the quality function are, for example, biophysical properties such as the rigidity or the curvature of the DNA sequence. Depending on the area of use it is also possible to include criteria which are associated with further DNA sequences. For example it is crucial in the area of DNA vaccination that the sequences used for vaccination show no significant similarity to the pathogenic elements of the natural viral genome, in order to reliably preclude unwanted recombination events. In the same way, vectors used for gene therapy purposes ought to show minimal similarity to sequences of the human genome in order firstly to preclude homologous recombination into the human genome and secondly to avoid vital genes being selectively switched off in transcription through RNA interference phenomena (RNAI phenomena). The latter is also of general importance in the production of recombinant cell factories and, in particular, in transgenic organisms.

The various criterion weights for various criteria can according to the invention be included differently in the overall weight function. In this connection the difference which can be maximally achieved through the corresponding criteria in the value of the quality function is important for the test sequence formed. However, a large proportion of certain criterion weights have DNA bases which cannot be changed by different CDS, such as, for example, the nucleotides in front of the CDS, which are also included in the calculation of the average GC content, and the nucleotides which are unaltered within synonymous codons. The individual weighting of a criterion vis-à-vis other criteria can therefore be made dependent on how greatly the quality of the test sequence differs from the target. It may be worthwhile to split up the criterion weights for further processing in mathematical functions for calculating the quality function into a part which is a measure of the portion of a criterion which is variable on use of different CDS, and a part which is a measure of the unaltered portions.

The embodiments of the invention which are described above are explained further below with reference to two specific examples.

Example 1

The intention is to ascertain the optimal DNA sequence pertaining to the (fictional) amino acid sequence AASeq1 from below. A conventional back-translation with optimization for optimal codon usage serves as reference.

AASeq1:

ASSeq1:

1	2	3	4	5	6	7	8	9	10	11	12	13	14
E__	Q__	F__	I__	I__	K__	N__	M__	F__	I__	I__	K__	N__	A__
GAA	CAG	TTT	ATT	ATT	AAA	AAC	ATG	TTT	ATT	ATT	AAA	AAC	GCG
GAG	CAA	TTC	ATC	ATC	AAG	AAT		TTC	ATC	ATC	AAG	AAT	GCC
			ATA	ATA					ATA	ATA			GCA
													GCT

The optimization is based on the following criteria:

- the codon usage is to be optimized to the codon usage of E. Coli K12.
- the GC content is to be as close as possible to 50%.
- repetitions are to be excluded as far as possible
- the Nla III recognition sequence CATG is to be excluded

The assessment function used for the codon usage is the following function:

$$CUScore = \langle CU \rangle$$

where <CU> in this example is the arithmetic mean of the relative adaptiveness over the codon positions of the test sequence.

To represent the codon usage of a codon, for better comparability of the codon quality of different amino acids, the best codon in each case for a particular amino acid is set equal to 100, and the worse codons are rescaled according to their tabulated percentage content. A CUScore of 100 therefore means that only the codons optimal for E. Coli K12 are used.

The weight for the percentage GC content is calculated as follows:

$$GCScore = |\langle GC\rangle - GC_{desire}|^{1.3} \times 0.8$$

To ascertain the individual weights of the alignments (alignment score), an optimal local alignment of the test sequence with a part region of the test sequence which includes a maximum of the last 36 bases of the complete test sequence is generated with exclusion of the identity alignment (alignment of the complete part region with itself) (cf. fig. 3, 4a, 4b).

The assessment parameter for a base position used in this case for calculating the dynamic programming matrix are:

Match = 1;
Mismatch = -2;
Gap = -2.

The corresponding criterion weight is specified by a power of the optimal alignment score in the examined region of the test sequence:

$$REPScore = (Score_{alignment})^{1.3}$$

A site score of 100 000 is allocated for each CATG sequence found.

The overall quality function TotScore results

$$TotScore = CUScore - GCScore - REPScore - SiteScore$$

The CDS length m is 3 codons (9 bases).

An optimization only for optimal codon usage results in the following sequence:

1	2	3	4	5	6	7	8	9	10	11	12	13	14
E__	Q__	F__	I__	I__	K__	N__	M__	F__	I__	I__	K__	N__	A__
GAA	**CAG**	**TTT**	**ATT**	**ATT**	**AAA**	**AAC**	**ATG**	**TTT**	**ATT**	**ATT**	**AAA**	**AAC**	**GCG**

It is characterized by the following properties:

- highly repetitive, caused by the amino acid sequence F_I_I_K_N which appears twice (the repetitive sequence with the highest score (18) is shown):

```
19 AACATGTTTATTATTAAAAAC
   |||| ||||||||||||||||
 2 AACA-GTTTATTATTAAAAAC
```

- GC content: 21.4%
- the Nla III recognition sequence CATG is present
- average codon usage: 100

If the optimization is carried out according to the algorithm of the invention with the abovementioned assessment functions and parameters, the following DNA sequence is obtained:

1	2	3	4	5	6	7	8	9	10	11	12	13	14
E__	Q__	F__	I__	I__	K__	N__	M__	F__	I__	I__	K__	N__	A__
GAA	**CAG**	**TTC**	**ATC**	**ATC**	**AAA**	**AAT**	**ATG**	**TTT**	**ATT**	**ATC**	**AAG**	**AAC**	**GCG**

It is characterized by the following properties:

- scarçely repetitive (the alignment shown below with the highest contribution has a score of 6)

```
11 TCATCA
   ||||||
 8 TCATCA
```

- GC content: 31.0%
- the Nla III recognition sequence CATG has been avoided
- average codon usage: 88

In the optimization result according to the invention, the codon optimal in relation to codon usage was not chosen at five amino acid positions. However, the sequence found a represents an optimal balance of the various requirements in terms of codon usage, GC content and ideal sequence properties (avoidance of repetitions).

For the amino acids with the numbers 3, 4, 5, the higher GC content of the codons which are worse in terms of codon usage is the reason for the choice. At position 6, however, on comparison of the codons AAA and AAG, the considerably better codon usage of the AAA codon is dominant, although choice of the AAG codon would lead to a better GC score. On formation of the CDS at base position 13, the codon AAC is preferred for amino acid No. 7 since, with a window size of 3 codons for the CDS, it is not yet evident that this choice will lead to the formation of the CATG DNA motif which is to be avoided (the genetic code is not degenerate for methionine, i.e. there is only one codon for expression of methionine). In the formation of the CDS at base position 16, however, this has been recognized and consequently the codon AAT is chosen. Besides codon usage and GC content, also the avoidance of a repetitive DNA sequence plays in the choice of the codon for amino acids 9 to 13. Because of the identical amino acid sequences of amino acids Nos. 3 to 7 and 9 to 13 a crucial role. For this reason, the codons TTT and ATT are preferred for amino acids 9 and 10, in contrast to previously (Aad. 3,4).

The following table illustrates the individual steps of the algorithm which have led to the optimization result indicated above. It enables the progress of the algorithm to be understood step by step. Moreover, all combination DNA sequences (CDS) formed by the software are listed in detail for each starting position.

The following information is given for each possible CDS:

- the test sequence which was formed from each CDS and the previously optimized DNA sequence which is used for evaluating the CDS,
- the scores which were ascertained for codon usage, GC content, repetitiveness and DNA sites found (CU, GC, Rep, Site)
- the repetitive element with the highest alignment score ascertained for the particular test sequence,
- the total score ascertained.

The CDS are in this case arranged according to decreasing total score, i.e. the first codon of the first CDS shown is attached to the previously optimized DNA sequence.

CDS starting position 1 for amino acid 1 E

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
GAACAGTTC GAACAGTTC	92	5	0	0,0		87,0
GAACAGTTT GAACAGTTT	100	19	0	0,0		81,0
GAGCAGTTT GAGCAGTTT	82	5	0	0,0		77,0
GAGCAGTTC GAGCAGTTC	73	5	0	0,0		68,0
GAACAATTC GAACAATTC	76	19	0	0,0		57,0
GAGCAATTC GAGCAATTC	58	5	0	0,0		53,0
GAACAATTT GAACAATTT	85	38	0	0,0		47,0
GAGCAATTT GAGCAATTT	66	19	0	0,0		47,0

CDS starting position 4 for amino acid 2 Q

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
CAGTTCATC GAACAGTTCATC	86	8	0	0,0		78,0
CAGTTTATC GAACAGTTTATC	94	19	0	0,0		75,0
CAGTTCATT GAACAGTTCATT	92	19	0	0,0		73,0
CAGTTTATT GAACAGTTTATT	100	33	0	0,0		67,0
CAATTCATC GAACAATTCATC	70	19	0	0,0		51,0
CAATTTATC GAACAATTTATC	79	33	0	0,0		46,0
CAGTTCATA GAACAGTTCATA	63	19	0	0,0		44,0
CAATTCATT GAACAATTCATT	76	33	0	0,0		43,0
CAGTTTATA GAACAGTTTATA	71	33	0	0,0		38,0
CAATTTATT GAACAATTTATT	85	48	0	0,0		37,0
CAATTCATA GAACAATTCATA	48	33	C	0,0		15,0
CAATTTATA GAACAATTTATA	56	48	0	0,0		8,0

CDS starting position 7 for amino acid 3 F

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
TTCATCATC GAACAGTTCATCATC	80	10	0	0,0		70,0

TTTATCATC GAACAGTTTATCATC	88	19	0	0,0		69,0
TTCATTATC GAACAGTTCATTATC	86	19	0	0,0		67,0
TTCATCATT GAACAGTTCATCATT	86	19	0	0,0		67,0
TTTATTATC GAACAGTTTATTATC	94	30	0	0,0		64,0
TTTATCATT GAACAGTTTATCATT	94	30	0	0,0		64,0
TTCATTATT GAACAGTTCATTATT	92	30	0	0,0		62,0
TTTATTATT GAACAGTTTATTATT	100	42	0	0,0		58,0
TTCATCATA GAACAGTTCATCATA	57	19	0	0,0		38,0
TTCATAATC GAACAGTTCATAATC	57	19	0	0,0		38,0
TTTATCATA GAACAGTTTATCATA	65	30	0	0,0		35,0
TTTATAATC GAACAGTTTATAATC	65	30	0	0,0		35,0
TTCATTATA GAACAGTTCATTATA	63	30	0	0,0		33,0
TTCATAATT GAACAGTTCATAATT	63	30	0	0,0		33,0
TTTATTATA GAACAGTTTATTATA	71	42	0	0,0		29,0
TTTATAATT GAACAGTTTATAATT	71	42	0	0,0		29,0
TTCATAATA GAACAGTTCATAATA	34	30	0	0,0		4,0
TTTATAATA GAACAGTTTATAATA	43	42	0	0,0		1,0

CDS starting position 10 for amino acid 4 I

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
ATCATCAAA GAACAGTTCATCATCAAA	88	19	0	0,0		69,0
ATTATCAAA GAACAGTTCATTATCAAA	94	28	0	0,0		66,0
ATCATTAAA GAACAGTTCATCATTAAA	94	28	0	0,0		66,0
ATTATTAAA GAACAGTTCATTATTAAA	100	38	0	0,0		62,0
ATCATCAAG GAACAGTTCATCATCAAG	65	11	0	0,0		54,0
ATTATCAAG GAACAGTTCATTATCAAG	71	19	0	0,0		52,0
ATCATTAAG GAACAGTTCATCATTAAG	71	19	0	0,0		52,0
ATTATTAAG GAACAGTTCATTATTAAG	77	28	0	0,0		49,0

ATCATAAAA GAACAGTTCATCATAAAA	65	28	0	0,0	TCAT TCAT	37,0
ATAATCAAA GAACAGTTCATAATCAAA	65	28	0	0,0	TCA TCA	37,0
ATTATAAAA GAACAGTTCATTATAAAA	71	38	0	0,0	AAA AAA	33,0
ATAATTAAA GAACAGTTCATAATTAAA	71	38	0	0,0	TAA TAA	33,0
ATCATAAAG GAACAGTTCATCATAAAG	43	19	0	0,0	TCAT TCAT	24,0
ATAATCAAG GAACAGTTCATAATCAAG	43	19	0	0,0	TCA TCA	24,0
ATTATAAAG GAACAGTTCATTATAAAG	49	28	0	0,0	AA AA	21,0
ATAATTAAG GAACAGTTCATAATTAAG	49	28	0	0,0	TAA TAA	21,0
ATAATAAAA GAACAGTTCATAATAAAA	43	38	0	0,0	ATAA ATAA	5,0
ATAATAAAG GAACAGTTCATAATAAAG	20	28	0	0,0	ATAA ATAA	-8,0

CDS starting position 13 for amino acid 5 I

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
ATCAAAAAC GAACAGTTCATCATCAAAAAC	94	19	0	0,0	TCATCA TCATCA	75,0
ATTAAAAAC GAACAGTTCATCATTAAAAAC	100	27	0	0,0	TCAT TCAT	73,0
ATCAAAAAT GAACAGTTCATCATCAAAAAT	88	27	0	0,0	TCATCA TCATCA	61,0
ATTAAAAAT GAACAGTTCATCATTAAAAAT	94	35	0	0,0	TCAT TCAT	59,0
ATTAAGAAC GAACAGTTCATCATTAAGAAC	77	19	0	0,0	GAAC GAAC	58,0
ATCAAGAAC GAACAGTTCATCATCAAGAAC	71	13	0	0,0	TCATCA TCATCA	58,0
ATCAAGAAT GAACAGTTCATCATCAAGAAT	65	19	0	0,0	TCATCA TCATCA	46,0
ATTAAGAAT GAACAGTTCATCATTAAGAAT	71	27	0	0,0	TCAT TCAT	44,0
ATAAAAAAC GAACAGTTCATCATAAAAAAC	71	27	0	0,0	TCAT-A-AAAAA TCATCATAAAAA	44,0
ATAAAAAAT GAACAGTTCATCATAAAAAAT	65	35	0	0,0	TCAT-A-AAAAA TCATCATAAAAA	30,0
ATAAAGAAC GAACAGTTCATCATAAAGAAC	49	19	0	0,0	GAAC GAAC	30,0
ATAAAGAAT GAACAGTTCATCATAAAGAAT	43	27	0	0,0	TCAT TCAT	16,0

CDS starting position 16 for amino acid 6 K

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
AAAAATATG GAACAGTTCATCATCAAAAATATG	94	26	0	0,0	TCATCA TCATCA	68,0

AAGAATATG GAACAGTTCATCATCAAGAATATG	71	19	0	0,0	TCATCA TCATCA	52,0
AAAAACATG GAACAGTTCATCATCAAAAACATG	100	19	200000	0,0	TCATCA TCATCA	919,0
AAGAACATG GAACAGTTCATCATCAAGAACATG	77	13	200000	0,0	TCATCA TCATCA	936,0

CDS starting position 19 for amino acid 7 N

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
AATATGTTT GAACAGTTCATCATCAAAAATATGTTT	94	35	0	0,0	TCATCA TCATCA	59,0
AATATGTTC GAACAGTTCATCATCAAAAATATGTTC	86	28	0	0,0	TCATCA TCATCA	58,0
AACATGTTT GAACAGTTCATCATCAAAAACATGTTT	100	28	200000	0,0	TCATCA TCATCA	928,0
AACATGTTC GAACAGTTCATCATCAAAAACATGTTC	92	21	200000	0,0	AACATGTTC AACA-GTTC	929,0

CDS starting position 22 for amino acid 8 M

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
ATGTTTATC GAACAGTTCATCATCAAAAATATGTTTATC	94	35	0	0,0	TCATCA TCATCA	59,0
ATGTTTATT GAACAGTTCATCATCAAAAATATGTTTATT	100	42	0	0,0	TCATCA TCATCA	58,0
ATGTTCATT GAACAGTTCATCATCAAAAATATGTTCATT	92	35	0	0,0	GTTCAT GTTCAT	57,0
ATGTTCATC GAACAGTTCATCATCAAAAATATGTTCATC	86	28	0	12,5	GTTCATC GTTCATC	45,0
ATGTTTATA GAACAGTTCATCATCAAAAATATGTTTATA	71	42	0	0,0	TCATCA TCATCA	29,0
ATGTTCATA GAACAGTTCATCATCAAAAATATGTTCATA	63	35	0	0,0	GTTCAT GTTCAT	28,0

CDS starting position 25 for amino acid 9 F

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
TTTATTATC GAACAGTTCATCATCAAAAATATGTTTATTATC	94	42	0	0,0	TCATCA TCATCA	52,0
TTTATCATT GAACAGTTCATCATCAAAAATATGTTTATCATT	94	42	0	0,0	TCATCA TCATCA	52,0
TTCATTATT GAACAGTTCATCATCAAAAATATGTTCATTATT	92	42	0	0,0	GTTCAT GTTCAT	50,0
TTTATCATC GAACAGTTCATCATCAAAAATATGTTTATCATC	88	35	0	12,5	GTTTATCATC GTTCATCATC	40,0
TTTATTATT GAACAGTTCATCATCAAAAATATGTTTATTATT	100	49	0	12,5	TCATCA--AAAATATGTTTATTATT TCATCATCAAAA-ATATGT-TTATT	38,0
TTCATTATC GAACAGTTCATCATCAAAAATATGTTCATTATC	86	35	0	12,5	GTTCATTATC GTTCATCAT-	38,0
TTCATCATT GAACAGTTCATCATCAAAAATATGTTCATCATT	86	35	0	17,4	GTTCATCAT GTTCATCAT	34,0

TTCATCATC GAACAGTTCATCATCAAAAATATGTTCATCATC	80	28	0	20,0	GTTCATCATC GTTCATCATC	32,0
TTTATCATA GAACAGTTCATCATCAAAAATATGTTTATCATA	65	42	0	0,0	TCATCA TCATCA	23,0
TTTATAATC GAACAGTTCATCATCAAAAATATGTTTATAATC	65	42	0	0,0	TCATCA TCATCA	23,0
TTTATTATA GAACAGTTCATCATCAAAAATATGTTTATTATA	71	49	0	0,0	TCATCA TCATCA	22,0
TTTATAATT GAACAGTTCATCATCAAAAATATGTTTATAATT	71	49	0	0,0	TCATCA TCATCA	22,0
TTCATAATT GAACAGTTCATCATCAAAAATATGTTCATAATT	63	42	0	0,0	GTTCAT GTTCAT	21,0
TTCATTATA GAACAGTTCATCATCAAAAATATGTTCATTATA	63	42	0	0,0	GTTCAT GTTCAT	21,0
TTCATAATC GAACAGTTCATCATCAAAAATATGTTCATAATC	57	35	0	12,5	GTTCATAATC GTTCATCATC	9,0
TTCATCATA GAACAGTTCATCATCAAAAATATGTTCATCATA	57	35	0	17,4	GTTCATCAT GTTCATCAT	5,0
TTTATAATA GAACAGTTCATCATCAAAAATATGTTTATAATA	43	49	0	0,0	TCATCA TCATCA	-6,0
TTCATAATA GAACAGTTCATCATCAAAAATATGTTCATAATA	34	42	0	0,0	GTTCAT GTTCAT	-8,0

CDS starting position 28 for amino acid · 10 I

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
ATTATCAAA GAACAGTTCATCATCAAAAATATGTTTATTATCAAA	94	49	0	12,5	GTTTATTATCAAA GTTCATCATCAAA	32,0
ATCATTAAA GAACAGTTCATCATCAAAAATATGTTTATCATTAAA	94	49	0	12,5	GTTTATCATTAAA GTTCATCATCAAA	32,0
ATTATCAAG GAACAGTTCATCATCAAAAATATGTTTATTATCAAG	71	42	0	0,0	TCATCA TCATCA	29,0
ATCATTAAG GAACAGTTCATCATCAAAAATATGTTTATCATTAAG	71	42	0	0,0	TCATCA TCATCA	29,0
ATTATTAAA GAACAGTTCATCATCAAAAATATGTTTATTATTAAA	100	57	0	14,9	TCATCA--AAAATATGTTTATTATTA TCATCATCAAAA-ATATGT-TTATTA	28,0
ATCATCAAA GAACAGTTCATCATCAAAAATATGTTTATCATCAAA	88	42	0	20,0	GTTTATCATCAAA GTTCATCATCAAA	26,0
ATTATAAAA GAACAGTTCATCATCAAAAATATGTTTATTATAAAA	71	57	0	0,0	TCATCA TCATCA	14,0
ATAATTAAA GAACAGTTCATCATCAAAAATATGTTTATAATTAAA	71	57	0	0,0	TCATCA TCATCA	14,0
ATTATTAAG GAACAGTTCATCATCAAAAATATGTTTATTATTAAG	77	49	0	14,9	TCATCA--AAAATATGTTTATTATTA TCATCATCAAAA-ATATGT-TTATTA	13,0
ATCATCAAG GAACAGTTCATCATCAAAAATATGTTTATCATCAAG	65	35	0	17,4	GTTTATCATCAA GTTCATCATCAA	13,0
ATAATCAAA GAACAGTTCATCATCAAAAATATGTTTATAATCAAA	65	49	0	12,5	GTTTATAATCAAA GTTCATCATCAAA	3,0
ATCATAAAA GAACAGTTCATCATCAAAAATATGTTTATCATAAAA	65	49	0	14,9	GTTTATCAT-AAAA GTTCATCATCAAAA	1,0
ATAATCAAG GAACAGTTCATCATCAAAAATATGTTTATAATCAAG	43	42	0	0,0	TCATCA TCATCA	1,0
ATTATAAAG GAACAGTTCATCATCAAAAATATGTTTATTATAAAG	49	49	0	0,0	TCATCA TCATCA	0,0

ATAATTAAG GAACAGTTCATCATCAAAAATATGTTTATAATTAAC	49	49	0	0.0	TCATCA TCATCA	0,0
ATCATAAAG GAACAGTTCATCATCAAAAATATGTTTATCATAAAG	43	42	0	12,5	GTTTATCAT-AAA GTTCATCATCAAA	-12,0
ATAATAAAA GAACAGTTCATCATCAAAAATATGTTTATAATAAAA	43	57	0	0,0	TCATCA TCATCA	-14,0
ATAATAAAG GAACAGTTCATCATCAAAAATATGTTTATAATAAAG	20	49	0	0,0	TCATCA TCATCA	-29,0

CDS starting position 31 for amino acid 11 I

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
ATCAAGAAC GAACAGTTCATCATCAAAAATATGTTTATTATCAAGAAC	71	42	0	0,0	TCATCA TCATCA	29,0
ATTAAAAAC GAACAGTTCATCATCAAAAATATGTTTATTATTAAAAAC	100	57	0	14,9	TCATCA--AAAATATGTTTATTATTA TCATCATCAAAA-ATATGT-TTATTA	28,0
ATCAAAAAC GAACAGTTCATCATCAAAAATATGTTTATTATCAAAAAC	94	49	0	17,4	GTTTATTATCAAAAA GTTCATCATCAAAAA	28,0
ATTAAAAAT GAACAGTTCATCATCAAAAATATGTTTATTATTAAAAAT	94	64	0	14,9	TCATCA--AAAATATGTTTATTATTA TCATCATCAAAA-ATATGT-TTATTA	15,0
ATTAAGAAC GAACAGTTCATCATCAAAAATATGTTTATTATTAAGAAC	77	49	0	14,9	TCATCA--AAAATATGTTTATTATTA TCATCATCAAAA-ATATGT-TTATTA	13,0
ATCAAAAAT GAACAGTTCATCATCAAAAATATGTTTATTATCAAAAAT	88	57	0	20,0	GTTTATTATCAAAAAT GTTCATCATCAAAAAT	11,0
ATCAAGAAT GAACAGTTCATCATCAAAAATATGTTTATTATCAAGAAT	65	49	0	12,5	GTTTATTATCAAGAAT GTTCATCATCAAAAAT	3,0
ATAAAGAAC GAACAGTTCATCATCAAAAATATGTTTATTATAAAGAAC	49	49	0	0,0	TCATCA TCATCA	0,0
ATTAAGAAT GAACAGTTCATCATCAAAAATATGTTTATTATTAAGAAT	71	57	0	14,9	TCATCA--AAAATATGTTTATTATTA TCATCATCAAAA-ATATGT-TTATTA	-1,0
ATAAAAAAC GAACAGTTCATCATCAAAAATATGTTTATTATAAAAAAC	71	57	0	14,9	TCATCA--AAAATATGTTTATTA-TA-AAAAA TCATCATCAAAA-ATATGT-TTATTATAAAAA	-1,0
ATAAAAAAT GAACAGTTCATCATCAAAAATATGTTTATTATAAAAAAT	65	64	0	14,9	TCATCA--AAAATATGTTTATTA-TA-AAAAA TCATCATCAAAA-ATATGT-TTATTATAAAAA	-14,0
ATAAAGAAT GAACAGTTCATCATCAAAAATATGTTTATTATAAAGAAT	43	57	0	0,0	TCATCA TCATCA	-14,0

CDS starting position 34 for amino acid 12 K

CDS test sequence	CU	GC	Site	Rep	Alignment	Total Score
AAGAACGCG GAACAGTTCATCATCAAAAATATGTTTATTATCAAGAACGCG	77	28	0	0,0	TCATCA TCATCA	49,0
AAAAACGCG GAACAGTTCATCATCAAAAATATGTTTATTATCAAAAACGCG	100	35	0	17,4	GTTTATTATCAAAAA GTTCATCATCAAAAA	48,0
AAGAACGCC GAACAGTTCATCATCAAAAATATGTTTATTATCAAGAACGCC	69	28	0	0,0	TCATCA TCATCA	41,0
AAAAACGCC GAACAGTTCATCATCAAAAATATGTTTATTATCAAAAACGCC	92	35	0	17,4	GTTTATTATCAAAAA GTTCATCATCAAAAA	40,0
AAAAATGCG GAACAGTTCATCATCAAAAATATGTTTATTATCAAAAATGCG	94	42	0	20,0	GTTTATTATCAAAAAT GTTCATCATCAAAAAT	32,0
AAGAACGCA GAACAGTTCATCATCAAAAATATGTTTATTATCAAGAACGCA	63	35	0	0,0	TCATCA TCATCA	25,0
AAAAACGCA GAACAGTTCATCATCAAAAATATGTTTATTATCAAAAACGCA	86	42	0	17,4	GTTTATTATCAAAAA GTTCATCATCAAAAA	27,0

AAAAATGCC GAACAGTTCATCATCAAAAATATGTTTATTATCAAAAATGCC	86	42	0	20,0	GTTTATTATCAAAAAT GTTCATCATCAAAAAT	24,0
AAGAACGCT GAACAGTTCATCATCAAAAATATGTTTATTATCAAGAACGCT	59	35	0	0,0	TCATCA TCATCA	24,0
AAGAATGCG GAACAGTTCATCATCAAAAATATGTTTATTATCAAGAATGCG	71	35	0	12,5	GTTTATTATCAAGAAT GTTCATCATCAAGAAT	23,0
AAAAACGCT GAACAGTTCATCATCAAAAATATGTTTATTATCAAAAACGCT	81	42	0	17,4	GTTTATTATCAAAAA GTTCATCATCAAAAA	22,0
AAGAATGCC GAACAGTTCATCATCAAAAATATGTTTATTATCAAGAATGCC	63	35	0	12,5	GTTTATTATCAAGAAT GTTCATCATCAAAAAT	15,0
AAAAATGCA GAACAGTTCATCATCAAAAATATGTTTATTATCAAAAATGCA	80	49	0	20,0	GTTTATTATCAAAAAT GTTCATCATCAAAAAT	11,0
AAAAATGCT GAACAGTTCATCATCAAAAATATGTTTATTATCAAAAATGCT	75	49	0	20,0	GTTTATTATCAAAAAT GTTCATCATCAAAAAT	6,0
AAGAATGCA GAACAGTTCATCATCAAAAATATGTTTATTATCAAGAATGCA	57	42	0	12,5	GTTTATTATCAAGAAT GTTCATCATCAAAAAT	2,0
AAGAATGCT GAACAGTTCATCATCAAAAATATGTTTATTATCAAGAATGCT	53	42	0	12,5	GTTTATTATCAAGAAT GTTCATCATCAAAAAT	-2,0

Example 2

This example considers the optimization of GFP for expression in E. Coli.

Origin of the amino acid sequence:

```
DEFINITION  Aequorea victoria green-fluorescent protein mRNA, complete cds.
ACCESSION   M62654

MSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTFSYGVQCFSRYP
DHMKQHDFFKSAMPEGYVQERTIFYKDDGNYKSRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKMEYNYNSHNV
YIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMILLEFVT
AAGITHGMDELYK
```

Codon usage table used: Escherichia coli K12
Origin: codon usage database on www.kazusa.or.ip/codon

The meanings below are:

<CU> : average renormalized codon usage of the CDS (15 bases long)

<GC> : average percentage GC content of the last 35 bases of the test sequence

GC_{desire}: desired GC content

The size of the window on which the GC content was calculated for the graphical representation in fig. 5b to 8b was 40 bases

Fig 5a and 5b show the results for the quality function:

$$Score = \langle CU \rangle$$

Fig. 6a and 6b show the results for the quality function

$$Score = \langle CU \rangle - |\langle GC \rangle - GC_{desire}|^{1.3} \times 0.8$$

Fig. 7a and 7b show the results for the quality function

$$Score = \langle CU \rangle - |\langle GC \rangle - GC_{desire}|^{1.3} \times 1.5$$

Fig. 8a and 8b show the results for the quality function

$$Score = \langle CU \rangle - |\langle GC \rangle - GC_{desire}|^{1.3} \times 5$$

Figures 5 to 8 illustrate the influence of the different weighting of two optimization criteria on the optimization result. The aim is to smooth the GC content distribution over the sequence and approach the value of 50%. In the case shown in fig. 5a and 5b, optimization was only for optimal codon usage, resulting in a very heterogeneous GC distribution which in some cases differed greatly from the target content. In the case of fig. 6a and 6b there is an ideal conjunction of a smoothing of the GC content to a value around 50% with a good to very good codon usage. The cases of fig. 7a and 7b, and 8a and 8b, finally illustrate that although a further GC content optimization is possible, it is necessarily at the expense of a poor codon usage in places.

Example 3

The efficiency of the method of the invention is illustrated by the following exemplary embodiment in which expression constructs with adapted and RNA- and codon-optimized reading frames were prepared, and in which the respective expression of the protein was quantified.

Selected cytokine genes and chemokine genes from various organisms (human: IL15, GM-CSF and mouse: GM-CSF, MIP1alpha) were cloned into the plasmid pcDNA3.1(+) (Invitrogen) to prepare expression plasmids. The reading frames of the corresponding genes were optimized using a codon choice like that preferentially found in human and murine cells, respectively, and using the optimization method described herein for maximal expression in the relevant organism. The corresponding genes were artificially assembled after the amino acid sequence of the genes was initially translated into a nucleotide sequence like that calculated by the described method taking account of various parameters.

The optimization of the cytokine genes was based on the following parameters:

the following quality function was used to assess the test sequence:

TotScore = CUScore - GCScore - REPScore - SEKscore - SiteScore

The CDS length was 5 codons.

The individual scores are in this case defined as follows:

a) *CUScore = <CU>*

where <CU> represents the arithmetic mean of the relative adaptiveness values of the CDS codons, multiplied by 100, i.e. to represent the codon usage of a codon, for better comparability of the codon quality of different amino acids the codon which is best in each case for a particular amino acid is set equal to 100, and the worst codons are rescaled according to their tabulated percentage content. A *CUScore* of 100 therefore means that only codons optimal for the expression system are used. In the cytokine genes to be optimized, the CUScore was calculated on the basis of the codon frequencies in humans (*Homo sapiens*) which are listed in the table below. Only codons whose relative adaptiveness is greater than 0.6 are used in the optimizations.

AmAcid	Codon	Frequency
Ala	GCG	0.10
	GCA	0.23
	GCT	0.26
	GCC	0.40
Arg	AGG	0.20
	AGA	0.20
	CGG	0.20
	CGA	0.11
	CGT	0.08
	CGC	0.19
Asn	AAT	0.45
	AAC	0.55
Asp	GAT	0.46
	GAC	0.54
Cys	TGT	0.45
	TGC	0.55
End	TGA	0.61
	TAG	0.17
	TAA	0.21
Gln	CAG	0.73
	CAA	0.27
Glu	GAG	0.58
	GAA	0.42
Gly	GGG	0.25
	GGA	0.25
	GGT	0.16
	GGC	0.34
His	CAT	0.41
	CAC	0.59
Ile	ATA	0.18
	ATT	0.35
	ATC	0.47

AmAcid	Codon	Frequency
Leu	TTG	0.12
	TTA	0.08
	CTG	0.38
	CTA	0.09
	CTT	0.13
	CTC	0.20
Lys	AAG	0.56
	AAA	0.44
Met	ATG	1.00
Phe	TTT	0.45
	TTC	0.55
Pro	CCG	0.11
	CCA	0.27
	CCT	0.28
	CCC	0.34
Ser	AGT	0.15
	AGC	0.24
	TCG	0.05
	TCA	0.15
	TCT	0.18
	TCC	0.22
Thr	ACG	0.11
	ACA	0.29
	ACT	0.24
	ACC	0.37
Trp	TGG	1.00
Tyr	TAT	0.44
	TAC	0.56
Val	GTG	0.45
	GTA	0.12
	GTT	0.18
	GTC	0.24

Scource: GenBank release 138.0 [October 15 2003] codon usage database, http://www.kazusa.or.jp/codon/

b) $GCScore = |\langle GC\rangle - GC_{desire}| \times 2$

with <GC>: average percentage GC content of the last 35 bases of the test sequence

GC_{desire}: desired percentage GC content of 60%

c) $REPScore = (Score_{alignment,max})$

To ascertain the individual weights of the alignments (alignment score), a local alignment of a terminal part region of the test sequence which includes a maximum of the last 35 bases of the complete test sequence is carried out with the region located in front in the test sequence.

Assessment parameters used in this case for a base position are:

Match = 10;
Mismatch = -30;
Gap = -30.

The corresponding criterion weight *REPScore* is defined as the highest alignment score $Score_{alignment,maxt}$ reached in the checked region of the test sequence. If the value of $Score_{alignment,max}$) is < 100, then REPScore is set equal to 0.

d) *SEKScore* = ($Score_{InvAligne\ nl\ max)}$)

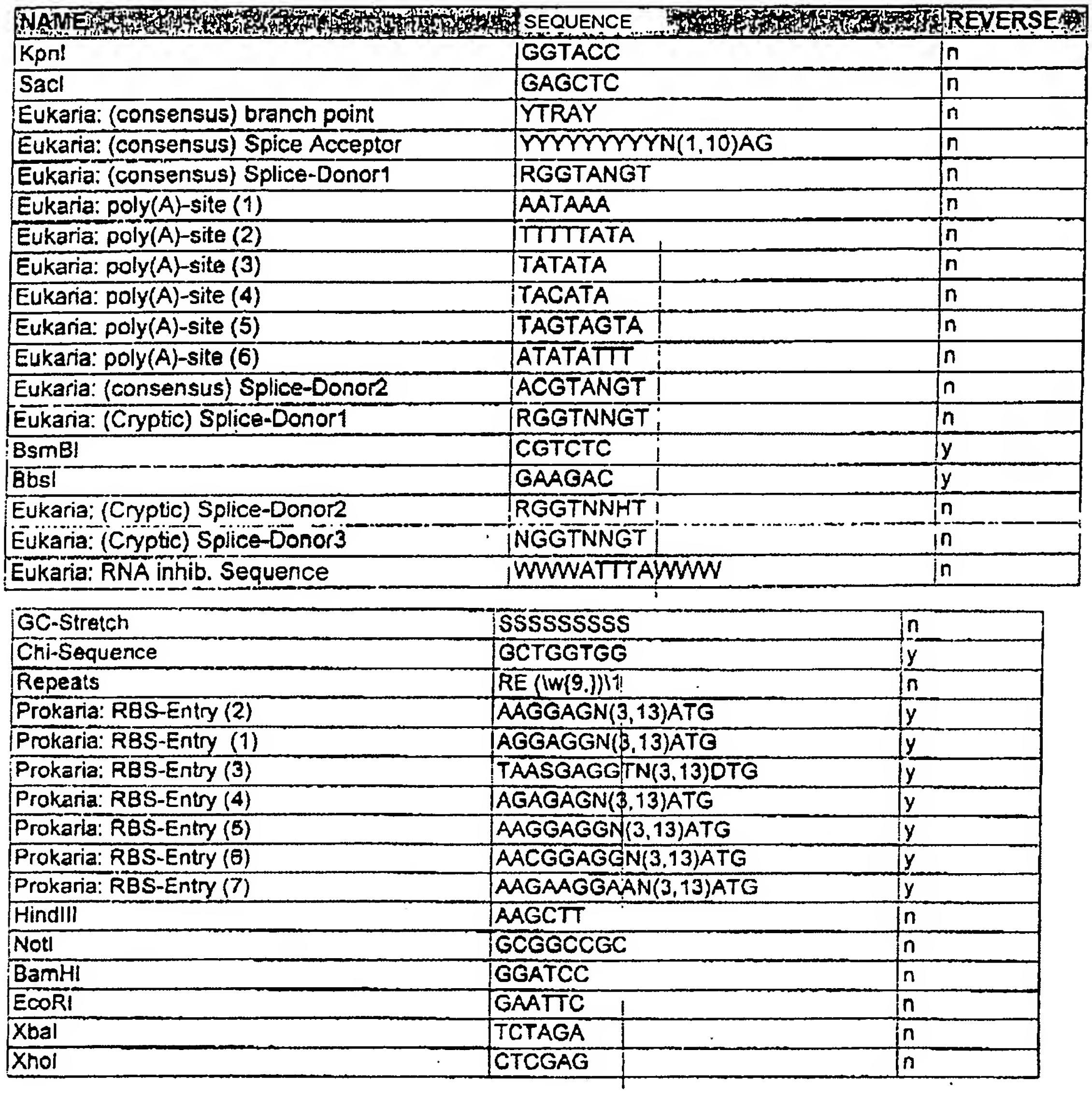
The criterion weight SEKScore weights inverse alignments in the sequence produced. To ascertain the individual weight of an alignment ($\mathrm{Score}_{\mathrm{InvAlignment,max}}$), a local alignment of the inverse complementary of the test sequence is carried out with the part region of the test sequence which includes a maximum of the last 35 bases of the complete test sequence.

The assessment parameters used for a base position in this case are:
Match = 10;
Mismatch = -30;
Gap = -30.

The corresponding criterion weight SEKScore is defined as the highest alignment score $Score_{InvAlignment,max}$ reached in the checked region of the test sequence. If the value of $Score_{InvAlignment,max}$ is < 100, then *SEKScore* is set equal to 0.

e) Sitescore

The following table lists the sequence motifs taking into account in ascertaining the SITEScore. Where a y appears on the heading "REVERSE", both the stated sequence motif and the relevant inverse complementary sequence motif was taken into account. If an n is indicated under this heading, only the stated sequence motif, but not the sequence motif inverse complementary thereto, was taken into account. For each occurrence of the sequence motifs listed in the table (or their inverse complementary if REVERSE = y) within the last 35 bases of the test sequence, the criterion weight *SITEScore* is increased by a value of 100 000.



NAME	SEQUENCE	REVERSE
KpnI	GGTACC	n
SacI	GAGCTC	n
Eukaria: (consensus) branch point	YTRAY	n
Eukaria: (consensus) Spice Acceptor	YYYYYYYYYN(1,10)AG	n
Eukaria: (consensus) Splice-Donor1	RGGTANGT	n
Eukaria: poly(A)-site (1)	AATAAA	n
Eukaria: poly(A)-site (2)	TTTTTATA	n
Eukaria: poly(A)-site (3)	TATATA	n
Eukaria: poly(A)-site (4)	TACATA	n
Eukaria: poly(A)-site (5)	TAGTAGTA	n
Eukaria: poly(A)-site (6)	ATATATTT	n
Eukaria: (consensus) Splice-Donor2	ACGTANGT	n
Eukaria: (Cryptic) Splice-Donor1	RGGTNNGT	n
BsmBI	CGTCTC	y
BbsI	GAAGAC	y
Eukaria: (Cryptic) Splice-Donor2	RGGTNNHT	n
Eukaria: (Cryptic) Splice-Donor3	NGGTNNGT	n
Eukaria: RNA inhib. Sequence	WWWATTTAWWW	n
GC-Stretch	SSSSSSSSS	n
Chi-Sequence	GCTGGTGG	y
Repeats	RE (\w{9,})\1	n
Prokaria: RBS-Entry (2)	AAGGAGN(3,13)ATG	y
Prokaria: RBS-Entry (1)	AGGAGGN(3,13)ATG	y
Prokaria: RBS-Entry (3)	TAASGAGGTN(3,13)DTG	y
Prokaria: RBS-Entry (4)	AGAGAGN(3,13)ATG	y
Prokaria: RBS-Entry (5)	AAGGAGGN(3,13)ATG	y
Prokaria: RBS-Entry (6)	AACGGAGGN(3,13)ATG	y
Prokaria: RBS-Entry (7)	AAGAAGGAAN(3,13)ATG	y
HindIII	AAGCTT	n
NotI	GCGGCCGC	n
BamHI	GGATCC	n
EcoRI	GAATTC	n
XbaI	TCTAGA	n
XhoI	CTCGAG	n

Appropriate unique restriction cleavage sites were introduced for subcloning. The complete nucleotide sequences are indicated in the annex. The sequences modified in this way were prepared as fully synthetic genes (Geneart, Regensburg). The resulting coding DNA fragments was placed under the transcriptional control of the cytomegalo virus (CMV) early promotor/enhancer in the expression vector pcDNA3.1(+) using the restriction cleavage sites *Hind*III and *Not*I. To prepare expression plasmids which are analogous but unaltered in their codon choice (wild-type reference constructs), the coding regions (c-DNA constructs were produced from RZPD) were cloned after PCR amplification with appropriate oligonucleotides likewise using the *Hind*III and *Not*I restriction cleavage sites in pcDNA3.1(+).

To quantify cytokine/chemokine expression, human cells were transfected with the respective expression constructs, and the amount of protein in the cells and in the cell culture supernatant was measured by using commercial ELISA test kits.

All the cell culture products were from Life Technologies (Karlsruhe). Mammalian cell lines were cultivated at 37°C and 5% CO_2. The human lung carcinoma cell line H1299 was cultivated in Dulbecco's modificated Eagle medium (DMEM) with L-glutamine, D-glucose (4.5 mg/ml), sodium pyruvate, 10% inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were subcultivated in the ratio 1:10 after reaching confluence.

2.5×10^5 cells were seeded in 6-well cell culture dishes and, after 24 h, transfected by calcium phosphate coprecipitation (Graham and Eb, 1973) with 15 µg of expression plasmids or pcDNA 3.1 vector (mock control). Cells and culture supernatants were harvested 48 h after the transfection. Insoluble constituents in the supernatants were removed by centrifugation and 10 000 ×g and 4°C for 10 min. The transfected cells were washed twice with ice-cold PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 ml NaCl, 2.7 mM KCl), detached with 0.05% trypsin/EDTA, centrifuged at 300 ×g for 10 min and lysed in 100 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS (w/v), 1% Nonidet P40 (v/v), 0.5% Na deoxycholate (w/v)) on ice for 30 min. Insoluble constituents of the cell lysate were removed by centrifugation at 10 000 ×g and 4°C for 30 min. The total amount of protein in the cell lysate supernatant was determined using the Bio-Rad protein assay (Bio-Rad, Munich) in accordance with the manufacturer's instructions.

The specific protein concentrations in the cell lysates and cell culture supernatants were quantified by ELISA tests (BD Pharmingen for IL15 and GM-CSF; R & D Systems for MIP1alpha). Appropriate amounts of total protein of the cell lysate (0.2 to 5 µg) and dilutions of the supernatant (undiluted to 1:200) were analyzed according to the manufacturer's instructions, and the total concentration was calculated by means of a calibration plot. Fig. 9 shows a representative calibration plot for calculating the murine MIP1alpha concentration. Recombinant murine MIP1alpha was adjusted in accordance with the manufacturer's instructions by serial two-fold dilutions to increasing concentrations and employed in parallel with the samples from the cell culture experiments in the MIP1alpha specific ELISA test. The concentrations (x axis) were plotted against the measured O.D. values (450 nm, y axis), and a regression line was calculated using MS Excel (the regression coefficient R^2 is indicated).

This was supplemented by carrying out a detection by Western blot analyses for suitable samples. For GM-CSF samples, total proteins were precipitated from in each case 1 ml of cell culture supernatant by Na DOC (sodium deoxycholate) and TCA (trichloroacetic acid) and resuspended in 60 µl of 1× sample buffer (Laemmli, 1970). 20 µl were employed for each of the analyses. For IL15 detection, 25 µg of total protein from cell lysates were used. The samples were heated at 95°C for 5 min, fractionated on a 15% SDS/polyacrylamide gel (Laemmli, 1970) electrotransferred to a nitrocellulose membrane (Bio-Rad) and analyzed with appropriate monoclonal antibodies (BD Pharmingen), detected using a secondary, AP (alkaline phosphatase)-coupled antibody and demonstrated by chromogenic staining. Fig. 12A to C show the expression analysis of the synthetic reading frame and of the wild-type reading frames. H1299 cells were transfected with the stated constructs, and the protein production was detected by conventional immunoblot analyses. In this case, fig. 12A shows the analysis of the cell culture supernatants after Na Doc/TCA precipitation of human GM-CSF transfected H1299 cells, fig. 12B shows the analysis of the cell culture supernatants after Na Doc/TCA precipitation of murine GM-CSF transfected H1299 cells, fig. 12C shows the analysis of the cell lysates from human IL15 transfected H1299 cells. Molecular weights (precision plus protein standard, Bio-Rad) and loading of the wild-type, synthetic and mock-transfected samples are indicated. Mock transfection corresponds to transfection with original pcDNA3.1 plasmid.

The following table summarizes the expression differences with averages of all ELISA-analyzed experiments. The data correspond to the percentage difference in the total amount of protein (total amount of protein in cell lysate and supernatant) related to the corresponding wild-type construct (wt corresponds to 100%).

Comparision of the total amounts of protein after transfection of wild-type vs. synthetic expression constructs

Construct	Organism	MW*	StdDev**	n=
GM-CSF	human	173%	53%	4
IL15	human	181%	37%	3
GM-CSF	mouse	127%	12%	2
MIP11alpha	mouse	146%	48%	2

* percentage average of the amount of protein from n experiments (in duplicate) related to the total amount of protein for the corresponding wild-type construct

** standard deviation

Fig. 10 shows in the form of a bar diagram the relative amount of protein in relation to the respective wild-type construct (corresponds to 100%) and illustrates the percentage increase in the total amount of protein after transvection of synthetic expression constructs compared with wild-type expression constructs. H1299 cells were transfected with 15 µg of the stated cytokine/chemokine constructs. The respective protein production was quantified by conventional ELISA tests in the cell culture supernatant and in the cell lysate by means of appropriate standard plots (see fig. 9). The ratio of the total amount of protein of synthetic to wild-type protein was calculated in each experiment (consisting of two independent mixtures) and indicated as percent of the total wild-type protein. The bars represent the average of four experiments for human GM-CSF, of three experiments for human IL15 and of two experiments for murine MIP1alpha and GM-CSF, in each case in independent duplicates. The error bars correspond to the standard deviation.

Fig. 11 depicts a representative ELISA analysis of the cell lysates and supernatants of transfected H1299 cells for human GM-CSF. H1299 cells were transfected with 15 µg each of wild-type and optimized human GM-CSF contructs. The respective protein concentration was quantified by conventional ELISA tests in the cell culture supernatant and in the cell lysate by means of appropriate standard plots. The bars represent the value of the total amount of protein in the cell lysate (CL), in the cell culture supernatant (SN) and the total of these values (total) for in each case 2 independent mixtures (1 and 2).

This analysis shows that the increase in expression after optimization (hu GM-CSF opt) is consistently detectable in the cell lysate and supernatant. It also illustrates by way of example that secretion of the cytokines is unaffected by the optimization by this method. A distinct and reproducible increase in protein expression was detectable for all optimized constructs, with the synthesis efficiencies of the optimized genes being improved by comparison with the wild-type genes in each individual experiment.

Expression was additionally checked in Western blot analyses (fig. 12 A to C). Human and murine GM-CSF were detectable in the cell culture supernatant (after Na DOC/TCA precipitation) (fig. 12A and B), while human IL15 was detectable in the cell lysates (fig. 12C). The proteins were analyzed, compared with commercially available recombinant proteins (BD) and the molecular weight was correspondingly confirmed. It was not possible in these transient transfection experiments to detect murine MIP1alpha by immunoblot staining. Comparison of the wild-type with the synthetic proteins in these representative immunoblots confirms the data of the ELISA analyses of an improved protein synthesis through multiparameter optimization of these genes.

The features disclosed in the claims, the drawings and the description may be essential both singly and in any combination for implementation of the invention in its various embodiments.

Annex: SEQ-IDs and alignments of the DNA sequences used

SEQ-ID of. the indicated constructs:

SEQ-ID1 (human GM-CSF wild type):

```
  1 atgtggctgc agagcctgct gctcttgggc actgtggcct gcagcatctc tgcacccgcc
 61 cgctcgccca gccccagcac gcagccctgg gagcatgtga atgccatcca ggaggcccgg
121 cgtctcctga acctgagtag agacactgct gctgagatga atgaaacagt agaagtcatc
181 tcagaaatgt ttgacctcca ggagccgacc tgcctacaga cccgcctgga gctgtacaag
241 cagggcctgc ggggcagcct caccaagctc aagggcccct tgaccatgat ggccagccac
301 tacaagcagc actgccctcc aaccccggaa acttcctgtg caacccagat tatcaccttt
361 gaaagtttca aagagaacct gaaggacttt ctgcttgtca tcccctttga ctgctgggag
421 ccagtccagg agtag
```

SEQ-ID2 (human GM-CSF optimized):

```
  1 atgtggctgc agagcctgct gctgctggga acagtggcct gtagcatctc tgcccctgcc
 61 agaagcccta gccctagcac acagccttgg gagcacgtga atgccatcca ggaggccagg
121 agactgctga acctgagcag agatacagcc gccgagatga acgagaccgt ggaggtgatc
181 agcgagatgt tcgacctgca ggagcctaca tgcctgcaga cccggctgga gctgtataag
241 cagggcctga gaggctctct gaccaagctg aagggccccc tgacaatgat ggccagccac
301 tacaagcagc actgccctcc taccectgag acaagctgcg ccacccagat catcaccttc
361 gagagcttca aggagaacct gaaggacttc ctgctggtga tccccttcga ttgctgggag
421 cccgtgcagg agtag
```

SEQ-ID3 (human IL15 wild type):

```
  1 atgagaattt cgaaaccaca tttgagaagt atttccatcc agtgctactt gtgtttactt
 61 ctaaacagtc attttctaac tgaagctggc attcatgtct tcattttggg ctgtttcagt
121 gcagggcttc ctaaaacaga agccaactgg gtgaatgtaa taagtgattt gaaaaaaatt
181 gaagatctta ttcaatctat gcatattgat gctactttat atacggaaag tgatgttcac
241 cccagttgca aagtaacagc aatgaagtgc tttctcttgg agttacaagt tatttcactt
301 gagtccggag atgcaagtat tcatgataca gtagaaaatc tgatcatcct agcaaacaac
361 agtttgtctt ctaatggaa tgtaacagaa tctggatgca aagaatgtga ggaactggag
421 gaaaaaaata ttaaagaatt tttgcagagt tttgtacata ttgtccaaat gttcatcaac
481 acttcttag
```

SEQ-ID4 (human IL15 optimized):

```
  1 atgcggatca gcaagcccca cctgaggagc atcagcatcc agtgctacct gtgcctgctg
 61 ctgaacagcc acttcctgac agaggccggc atccacgtgt ttatcctggg ctgcttctct
121 gccggcctgc ctaagacaga ggccaactgg gtgaacgtga tcagcgacct gaagaagatc
181 gaggacctga tccagagcat gcacatcgac gccaccctgt acacagagag cgacgtgcac
241 cctagctgta aggtgaccgc catgaagtgc ttcctgctgg agctgcaggt gatcagcctg
301 gagagcggcg atgccagcat ccacgacacc gtggagaacc tgatcatcct ggccaacaac
361 agcctgagca gcaacggcaa tgtgaccgag agcggctgca aggagtgtga ggagctggag
421 gagaagaaca tcaaggagtt cctgcagagc ttcgtgcaca tcgtgcagat gttcatcaac
481 accagctag
```

SEQ-ID5 (murine GM-CSF wild type):

```
  1 atgtggctgc agaatttact tttcctgggc attgtggtct acagcctctc agcacccacc
 61 cgctcaccca tcactgtcac ccggccttgg aagcatgtag aggccatcaa agaagccctg
121 aacctcctgg atgacatgcc tgtcacattg aatgaagagg tagaagtcgt ctctaacgag
181 ttctccttca agaagctaac atgtgtgcag acccgcctga agatattcga gcagggtcta
241 cggggcaatt tcaccaaact caagggcgcc ttgaacatga cagccagcta ctaccagaca
301 tactgccccc caactccgga aacggactgt gaaacacaag ttaccaccta tgcggatttc
361 atagacagcc ttaaaacctt tctgactgat atcccctttg aatgcaaaaa accaggccaa
421 aaatag.
```

SEQ-ID6 (murine GM-CSF optimized):

```
  1 atgtggctgc agaacctgct gttcctgggc atcgtggtgt acagcctgag cgcccccacc
 61 aggagcccca tcaccgtgac caggccctgg aagcacgtgg aggccatcaa ggaggccctg
121 aacctgctgg acgacatgcc cgtgaccctg aacgaggagg tggaggtggt gagcaacgag
181 ttcagcttca agaagctgac ctgcgtgcag accaggctga agatcttcga gcagggcctg
```

```
241 aggggcaact tcaccaagct gaagggcgcc ctgaacatga ccgccagcta ctaccagacc
301 tactgccccc ccacccccga gaccgactgc gagacccagg tgaccaccta cgccgacttc
361 atcgacagcc tgaagacctt cctgaccgac atccccttcg agtgcaagaa gcccggccag
421 aagtag
```

SEQ-ID7 (murine MIPlapha wild type):

```
  1 atgaaggtct ccaccactgc ccttgctgtt cttctctgta ccatgacact ctgcaaccaa
 61 gtcttctcag cgccatatgg agctgacacc ccgactgcct gctgcttctc ctacagccgg
121 aagattccac gccaattcat cgttgactat tttgaaacca gcagcctttg ctcccagcca
181 ggtgtcattt tcctgactaa gagaaaccgg cagatctgcg ctgactccaa agagacctgg
241 gtccaagaat acatcactga cctggaactg aatgcctag
```

SEQ-ID8 (murine MIPlapha optimized):

```
  1 atgaaggtga gcaccacagc tctggctgtg ctgctgtgca ccatgaccct gtgcaaccag
 61 gtgttcagcg ctccttacgg cgccgatacc cctacagcct gctgcttcag ctacagcagg
121 aagatcccca ggcagttcat cgtggactac ttcgagacca gcagcctgtg ttctcagccc
181 ggcgtgatct tcctgaccaa gcggaacaga cagatctgcg ccgacagcaa ggagacatgg
241 gtgcaggagt acatcaccga cctggagctg aacgcctag
```

Alignments of the DNA sequences used

1. Human GM-CSF:

Upper line: SEQ-ID1 (human GM-CSF wild type), from 1 to 435

Lower line: SEQ-ID2 (human GM-CSF optimized), from 1 to 435

Wild type: optimized identity = 83.45% (363/435) gap = 0.00% (0/435)

```
1       ATGTGGCTGCAGAGCCTGCTGCTCTTGGGCACTGTGGCCTGCAGCATCTCTGCACCCGCC
        |||||||||||||||||||||||  |||| || |||||||| ||||||||||| || |||
1       ATGTGGCTGCAGAGCCTGCTGCTGCTGGGAACAGTGGCCTGTAGCATCTCTGCCCCTGCC

61      CGCTCGCCCAGCCCCAGCACGCAGCCCTGGGAGCATGTGAATGCCATCCAGGAGGCCCGG
         |    || ||||| ||||| ||||| |||||||| ||||||||||||||||||||| ||
61      AGAAGCCCTAGCCCTAGCACACAGCCTTGGGAGCACGTGAATGCCATCCAGGAGGCCAGG

121     CGTCTCCTGAACCTGAGTAGAGACACTGCTGCTGAGATGAATGAAACAGTAGAAGTCATC
         | || ||||||||||| ||||| || || || |||||||| || || || || || |||
121     AGACTGCTGAACCTGAGCAGAGATACAGCCGCCGAGATGAACGAGACCGTGGAGGTGATC

181     TCAGAAATGTTTGACCTCCAGGAGCCGACCTGCCTACAGACCCGCCTGGAGCTGTACAAG
           || ||||| ||||| |||||||| || ||||| |||||||| ||||||||||| |||
181     AGCGAGATGTTCGACCTGCAGGAGCCTACATGCCTGCAGACCCGGCTGGAGCTGTATAAG

241     CAGGGCCTGCGGGGCAGCCTCACCAAGCTCAAGGGCCCCTTGACCATGATGGCCAGCCAC
        ||||||||| | |||   || |||||||| ||||||||| |||| |||||||||||||||
241     CAGGGCCTGAGAGGCTCTCTGACCAAGCTGAAGGGCCCCCTGACAATGATGGCCAGCCAC

301     TACAAGCAGCACTGCCCTCCAACCCCGGAAACTTCCTGTGCAACCCAGATTATCACCTTT
        |||||||||||||||||||| ||||| || ||   ||| || |||||||| ||||||||
301     TACAAGCAGCACTGCCCTCCTACCCCTGAGACAAGCTGCGCCACCCAGATCATCACCTTC

361     GAAAGTTTCAAAGAGAACCTGAAGGACTTTCTGCTTGTCATCCCCTTTGACTGCTGGGAG
        || || ||||| ||||||||||||||||| ||||| || |||||||| || |||||||||
361     GAGAGCTTCAAGGAGAACCTGAAGGACTTCCTGCTGGTGATCCCCTTCGATTGCTGGGAG

421     CCAGTCCAGGAGTAG
        || || |||||||||
421     CCCGTGCAGGAGTAG
```

2. Human IL15:

Upper line: SEQ-ID3 (human IL15 wild type), from 1 to 489

Lower line: SEQ-ID4 (human IL15 optimized), from 1 to 489

Wild type: optimized identity = 70.55% (345/489) gap = 0.00% (0/489)

```
1      ATGAGAATTTCGAAACCACATTTGAGAAGTATTTCCATCCAGTGCTACTTGTGTTTACTT
       ||| | ||    || || ||  |||| || ||   ||||||||||||| ||||  | || 
1      ATGCGGATCAGCAAGCCCCACCTGAGGAGCATCAGCATCCAGTGCTACCTGTGCCTGCTG

61     CTAAACAGTCATTTTCTAACTGAAGCTGGCATTCATGTCTTCATTTTGGGCTGTTTCAGT
       || ||||| || || || || || || || || || || || ||  |||||||| |||  |
61     CTGAACAGCCACTTCCTGACAGAGGCCGGCATCCACGTGTTTATCCTGGGCTGCTTCTCT

121    GCAGGGCTTCCTAAAACAGAAGCCAACTGGGTGAATGTAATAAGTGATTTGAAAAAAATT
       || || || ||||| ||||| |||||||||||||||| || || || ||  |||| || || 
121    GCCGGCCTGCCTAAGACAGAGGCCAACTGGGTGAACGTGATCAGCGACCTGAAGAAGATC

181    GAAGATCTTATTCAATCTATGCATATTGATGCTACTTTATATACGGAAAGTGATGTTCAC
       || || || || ||    ||||| || || || ||  | || || || ||  || || |||
181    GAGGACCTGATCCAGAGCATGCACATCGACGCCACCCTGTACACAGAGAGCGACGTGCAC

241    CCCAGTTGCAAAGTAACAGCAATGAAGTGCTTTCTCTTGGAGTTACAAGTTATTTCACTT
       || || || || || || || |||||||||||| ||  ||||| | || || ||    || 
241    CCTAGCTGTAAGGTGACCGCCATGAAGTGCTTCCTGCTGGAGCTGCAGGTGATCAGCCTG

301    GAGTCCGGAGATGCAAGTATTCATGATACAGTAGAAAATCTGATCATCCTAGCAAACAAC
       |||  ||| ||||| || || || || || || || || |||||||||||| || ||||||
301    GAGAGCGGCGATGCCAGCATCCACGACACCGTGGAGAACCTGATCATCCTGGCCAACAAC

361    AGTTTGTCTTCTAATGGGAATGTAACAGAATCTGGATGCAAAGAATGTGAGGAACTGGAG
       ||  ||      || || ||||| || ||     || ||||| || |||||||| ||||||
361    AGCCTGAGCAGCAACGGCAATGTGACCGAGAGCGGCTGCAAGGAGTGTGAGGAGCTGGAG

421    GAAAAAAATATTAAAGAATTTTTGCAGAGTTTTGTACATATTGTCCAAATGTTCATCAAC
       || || || || || || ||  ||||||| || || || || || || ||||||||||||
421    GAGAAGAACATCAAGGAGTTCCTGCAGAGCTTCGTGCACATCGTGCAGATGTTCATCAAC

481    ACTTCTTAG
       ||    |||
481    ACCAGCTAG
```

3. Murine GM-CSF:

Upper line: SEQ-ID5 (murine GM-CSF wild type), from 1 to 426

Lower line: SEQ-ID6 (murine GM-CSF optimized), from 1 to 426

Wild type: optimized identity = 80.75% (344/426) gap = 0.00% (0/426)

```
1     ATGTGGCTGCAGAATTTACTTTTCCTGGGCATTGTGGTCTACAGCCTCTCAGCACCCACC
      |||||||||||||||  | || ||||||||||||| ||||| ||||||||    || ||||||
1     ATGTGGCTGCAGAACCTGCTGTTCCTGGGCATCGTGGTGTACAGCCTGAGCGCCCCCACC

61    CGCTCACCCATCACTGTCACCCGGCCTTGGAAGCATGTAGAGGCCATCAAAGAAGCCCTG
       |    ||||||||| || ||| |||| ||||||||| || ||||||||||| || ||||||
61    AGGAGCCCCATCACCGTGACCAGGCCCTGGAAGCACGTGGAGGCCATCAAGGAGGCCCTG

121   AACCTCCTGGATGACATGCCTGTCACATTGAATGAAGAGGTAGAAGTCGTCTCTAACGAG
      ||||| ||||| |||||||| || ||  ||||| || ||||| || || ||    ||||||
121   AACCTGCTGGACGACATGCCCGTGACCCTGAACGAGGAGGTGGAGGTGGTGAGCAACGAG

181   TTCTCCTTCAAGAAGCTAACATGTGTGCAGACCCGCCTGAAGATATTCGAGCAGGGTCTA
      |||  |||||||||||| || || |||||||||| | |||||||| |||||||||||| || 
181   TTCAGCTTCAAGAAGCTGACCTGCGTGCAGACCAGGCTGAAGATCTTCGAGCAGGGCCTG

241   CGGGGCAATTTCACCAAACTCAAGGGCGCCTTGAACATGACAGCCAGCTACTACCAGACA
       ||||||| |||||||||| || ||||||||| ||||||||||| ||||||||||||||||| 
241   AGGGGCAACTTCACCAAGCTGAAGGGCGCCCTGAACATGACCGCCAGCTACTACCAGACC

301   TACTGCCCCCCAACTCCGGAAACGGACTGTGAAACACAAGTTACCACCTATGCGGATTTC
      ||||||||||| || || || || ||||| || || || || |||||||| || || |||
301   TACTGCCCCCCCACCCCCGAGACCGACTGCGAGACCCAGGTGACCACCTACGCCGACTTC

361   ATAGACAGCCTTAAAACCTTTCTGACTGATATCCCCTTTGAATGCAAAAAACCAGGCCAA
      || |||||||| || ||||| ||||| || |||||||| || ||||| || || ||||| 
361   ATCGACAGCCTGAAGACCTTCCTGACCGACATCCCCTTCGAGTGCAAGAAGCCCGGCCAG

421   AAATAG
      || |||
421   AAGTAG
```

4. Murine MIP1alpha:

Upper line: SEQ-ID7 (murine MIP1alpha wild type), from 1 to 279

Lower line: SEQ-ID8 (murine MIP1alpha optimized), from 1 to 279

Wild type: optimized identity = 78.49% (219/279) gap = 0.00% (0/279)

```
1      ATGAAGGTCTCCACCACTGCCCTTGCTGTTCTTCTCTGTACCATGACACTCTGCAACCAA
       ||||||||   |||||| || || ||||| || || || |||||||| || ||||||||
1      ATGAAGGTGAGCACCACAGCTCTGGCTGTGCTGCTGTGCACCATGACCCTGTGCAACCAG

61     GTCTTCTCAGCGCCATATGGAGCTGACACCCCGACTGCCTGCTGCTTCTCCTACAGCCGG
       || |||   || || || || || || ||||| || ||||||||||||  ||||||| ||
61     GTGTTCAGCGCTCCTTACGGCGCCGATACCCCTACAGCCTGCTGCTTCAGCTACAGCAGG

121    AAGATTCCACGCCAATTCATCGTTGACTATTTTGAAACCAGCAGCCTTTGCTCCCAGCCA
       ||||| ||  | || |||||||| ||||| || || ||||||||||| || || |||||
121    AAGATCCCCAGGCAGTTCATCGTGGACTACTTCGAGACCAGCAGCCTGTGTTCTCAGCCC

181    GGTGTCATTTTCCTGACTAAGAGAAACCGGCAGATCTGCGCTGACTCCAAAGAGACCTGG
       || || || |||||||| ||| | ||| | ||||||||||| |||  ||| ||||| |||
181    GGCGTGATCTTCCTGACCAAGCGGAACAGACAGATCTGCGCCGACAGCAAGGAGACATGG

241    GTCCAAGAATACATCACTGACCTGGAACTGAATGCCTAG
       || || || |||||||| |||||||| ||||| ||||||
241    GTGCAGGAGTACATCACCGACCTGGAGCTGAACGCCTAG
```